IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Wright, J.L.C.
Appln. No.	:	09/385,834
Filed:	:	August 30, 1999
Title	:	A Nutritional Supplement for Lowering Serum Triglyceride and Cholesterol Level
Grp./A.U.	:	1616
Examiner	:	S. N. Qazi
Docket No.	:	76891

DECLARATION PURSUANT TO 37 CFR § 1.132

I, H. STEPHEN EWART Ph.D., hereby declare that:

1. I am currently employed by Ocean Nutrition Canada Limited, the assignee of the above-identified application, in the capacity of Principal Research Scientist. I am currently responsible for the ongoing research project from which the above-identified patent application arose. Details of my education, employment in research, and my academic publications are set forth in Exhibit 1 hereto. In view of my education and my involvement with the research project relating to this application, I have extensive background and experience in the area of nutritional supplements for lowering triglyceride and cholesterol levels.

2. I have carefully reviewed this patent application, the Office Action mailed April 26, 2002, and the references cited therein.

3. Claims 1, 5-11, 34 and 39 of the instant application stand rejected as being obvious over U.S. Patent No. 5,770,749 to Kutney *et al.* and U.S. Patent No. 4,879,312 to Kamarei *et al.* The Examiner states that Kutney *et al.* teach that phytosterols are effective in lowering plasma cholesterol levels and that Kamarei *et al.* teach that a diet rich in omega-3 fatty acids has beneficial effects in humans, including a reduction in plasma cholesterol and triglyceride levels. The Examiner goes on to state that the present claims differ from the references in claiming a nutritional supplement by employing a combination of phytosterols and an omega-3 fatty acid. The Examiner therefore concludes that it would be obvious to one skilled

in the art at the time of the invention to employ phytosterols in combination with omega-3 fatty acids in compositions and methods for lowering cholesterol and triglycerides in the bloodstream of a subject, because these agents are known individually for the treatment of the same disorder.

4. It is very important to recognize that the present invention concerns not mere mixtures of sterols and omega-3 fatty acids but rather *esters* of a sterol and an omega-3 fatty acid. For the purposes of making nutritional supplements, there are very important differences between a mixture of a sterol and a fatty acid, and a composition wherein the sterol has been chemically joined to the fatty acid through ester linkage.

5. Mere mixtures of sterols and omega-3 fatty acids are not useful for making nutritional supplements at least in part because the free sterol does not dissolve well into the fatty acid. When mixed together, the sterol remains crystalline, and the particles merely become suspended in the omega-3 fatty acid oil, resulting in a grimy paste-like material. This paste cannot be added to food products without substantially degrading their aesthetic properties. For example, a mere pasty mixture of a sterol and a fatty acid could not be added to margarine, without unacceptably altering the appearance, texture, and flavour of the margarine. Similarly, a pasty mixture is difficult to microencapsulate, the preferred means of delivering the nutritional supplement in cake mixes, baked goods, ice cream, etc.

6. Such pastes are also very difficult to formulate into pharmaceutical compositions. For instance, the paste cannot be easily packaged in a capsule, which is the preferred single dosage format. Similarly, the pasty composition could not be packaged in a liquid form, which requires a homogenous oil.

7. The present invention overcomes the above-identified problems by chemically joining the sterol to the omega-3 fatty acid through an ester linkage. The sterol ester thereby produced is an oily, viscous liquid, suitable for introduction into food products and for packaging in capsules and the like. Three to four grams of this sterol ester can be dissolved in about 20 grams of margarine or other dietary fat source, without altering significantly the texture/taste profile of the product.

8. As discussed in detail in my Declaration filed on April 12, 2001, the sterol esters of omega-3 fatty acids of the invention are effective for lowering *both* cholesterol and triglyceride levels in the blood of animals. This is, in fact, a very surprising result due to the differing mechanisms of action of sterols and omega-3 fatty acids.

9. Phytosterols are not absorbed in the digestive tract to any great extent. This is acknowledged in the Kutney *et al.* reference at column 2, lines 6-7 where it is stated that phytosterols have no nutritional value to humans, i.e., the phytosterol does not get absorbed into the bloodstream. The mechanism by which phytosterol lowers blood cholesterol appears to involve inhibition of cholesterol absorption in the small intestine by competing with cholesterol at critical points in the uptake process.

10. In contrast, in order to effect a reduction in bloodstream *triglyceride* levels, omega-3 fatty acids must be absorbed from the intestinal lumen into the bloodstream. Fish oil omega-3 fatty acids must travel in the bloodstream to the liver where they modulate the activity of several enzymes of carbohydrate and lipid. The overall effect is the promotion of hepatic fatty acid oxidation and reduction of triacylglycerol synthesis, with a consequent reduction of triacylglyverol release into the circulation (see article cited in current patent application: Connor and Connor ,1997, Are fish oils beneficial in the prevention and treatment of coronary artery disease? Am. J. Clin. Nutr. 66 (suppl.): 1020S-1031S .).

11. Therefore, at the time of the invention, it was unknown whether this opposing requirement would be met. In particular, would the sterol component of the ester prevent the fatty acid from being absorbed into the bloodstream? While there are digestive enzymes in the intestinal lumen with esterase activity that could potentially free the fatty acid from its ester linkage with the sterol, the degree to which this would occur was unpredictable, and therefore, it was unclear whether sufficient of the omega-3 fatty acids would be released to have a significant impact on serum triglyceride levels.

12. Moreover, contrary to what is stated in Kamarei *et al.*, the preponderance of scientific evidence is that omega-3 fatty acids do not lower

cholesterol, and may actually increase it. Harris (1989) J. Lipid. Res. 30:785-807, discussed in the present patent application at page 7, lines 25-27, concluded that fish oil consumption (omega-3 fatty acids) results either in no change in serum cholesterol, or actually leads to an increase in LDL cholesterol. Similarly, a recently reported study found that EPA and DHA, the principal omega-3 fatty acids found in fish oil, *increased* LDL cholesterol levels (see Stalenhoef *et al.* (2000) The effect of concentrated N-3 fatty acids versus gemfibrozill on plasma lipoproteins, low density lipoprotein heterogeneity and oxidizability in patients with hypertriglyceridemia. Atherosclerosis 153:129-138, attached hereto as Exhibit 2).

13. LDL cholesterol is the form of blood cholesterol lowered by ingestion of sterols. Thus, irrespective of the above-described complications arising from the esterification of sterols with the omega-3 fatty acids, based on the totality of the available scientific literature, it would have been expected that the cholesterol-*increasing* effect of the omega-3 fatty acid might reduce or counteract the cholesterol-lowering effect of the sterol. It would not have been expected that the combination of the sterol and the omega-3 fatty acid, particularly in esterified form, would result in a reduction in both cholesterol and triglyceride levels, as disclosed in the present application.

14. I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

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July 10/2002
Date

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Education

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09/84 - 05/86	M.Sc. in Biology, Mount Allison University Sackville, New Brunswick Supervisor: Dr. W.R. Driedzic
09/79 - 04/83	B.Sc. (Honours) in Biology, Mount Allison University Sackville, New Brunswick

Employment in Research

04/01 – present	Principal Research Scientist Ocean Nutrition Canada Ltd. Halifax, NS
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10/93 - 03/96	Postdoctoral fellow, Division of Cell Biology Hospital for Sick Children Toronto, Ontario
05/86 - 07/87	Research assistant, Department of Biology Mount Allison University Sackville, New Brunswick

Honours and Awards

09/93 - 09/95	Hugh Sellers Postdoctoral Fellowship Banting and Best Diabetes Centre
1992	Merck Frosst - Canadian Biochemical Society Student Travel Award
01/88 - 01/91	Memorial University Graduate Student Fellowship Memorial University of Newfoundland
09/80 - 05/83	Wilkinson Scholarship Mount Allison University
09/79 - 05/80	Entrance Scholarship Mount Allison University

Teaching experience

09/87 - 12/92	Laboratory teaching assistant for biology and biochemistry courses at Memorial University Introductory Biochemistry, 3100 (6 semesters) Techniques in Biochemistry, 4211 (3 semesters) Introductory Biology (1 semester) Pharmacy Tutorial (1 semester)
09/84 - 05/86	Laboratory teaching assistant for biology courses at Mount Allison University Animal Physiology, 3210 (2 semesters) Metabolism, 3501 (1 semester) Cell Biology (1 semester)
09/80 - 04/83	Laboratory teaching assistant for biology and chemistry courses at Mount Allison University Analytical Chemistry (1 semester) Introductory Biology and Ecology (3 semesters)

Publications

Refereed papers

Curtis, J.M., Dennis, D., Waddell, D.S., MacGillivray T., Ewart, H.S. (2002) Determination of LKPNM content in bonito muscle hydrolysates by LC-MS. J. Agric. Food

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Professional Memberships

Canadian Institute of Food Science and Technology

American Diabetes Association

American Heart Association

Nova Scotia Institute of Science (Councillor)

EXHIBIT 2

ELSEVIER

ATHEROSCLEROSIS

Atherosclerosis 153 (2000) 129–138

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The effect of concentrated n-3 fatty acids versus gemfibrozil on plasma lipoproteins, low density lipoprotein heterogeneity and oxidizability in patients with hypertrygliceridemia

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Abstract

We evaluated in a double-blind randomized trial with a double-dummy design in 28 patients with primary hypertriglyceridemia, the effect of gemfibrozil (1200 mg/day) versus Omacor (4 g/day), a drug containing the n-3 fatty acids eicosapentaenoic (EPA) and docosahexaenoic acid (DHA), on lipid and lipoprotein levels, low density lipoprotein (LDL) subfraction profile and LDL oxidizability. Both Omacor and gemfibrozil therapy resulted in a similar significant decrease in serum triglyceride (TG), very low density lipoprotein (VLDL) triglyceride and VLDL cholesterol concentrations and an increase in high density lipoprotein (HDL) and LDL cholesterol concentrations. The increase in LDL cholesterol was due to a significant increase in cholesterol content of the relatively buoyant LDL subfractions LDL1, LDL2 and LDL3, whereas the relative contribution of the dense LDL subfractions LDL4 and LDL5 to total LDL tended to decrease. So, both therapies resulted in a more buoyant LDL subfraction profile, reflected by a significant increase of the value of parameter K (+10.3% on Omacor vs +26.5% on gemfibrozil therapy, gemfibrozil vs Omacor $P > 0.05$). Cu^{2+}-induced oxidation of LDL was measured by continuous monitoring of conjugated dienes. After 12 weeks of Omacor treatment LDL appeared more prone to oxidative modification in vitro than LDL after gemfibrozil treatment, as measured by the significantly decreased lag time, preceding the onset of the lipid peroxidation. In both groups the rate of oxidation did not change with therapy. The amount of dienes formed during oxidation increased significantly on Omacor treatment, but not on gemfibrozil treatment. Plasma thiobarbituric acid reactive substances were higher after Omacor and lower after gemfibrozil treatment, although not significantly. We conclude that both Omacor and gemfibrozil have favorable effects on lipid and lipoprotein concentrations and the LDL subfraction profile. However, Omacor increased the susceptibility of LDL to oxidation, whereas gemfibrozil did not affect the resistance of LDL to oxidative modification in vitro. The clinical relevance of these changes remains to be established in the light of other postulated favorable effects of n-3 fatty acids on the course of cardiovascular disease. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Fish oil; Gemfibrozil; Omega-3 fatty acids; Hypertriglyceridemia; Low density lipoprotein subfractions; Low density lipoprotein oxidation

1. Introduction

Subjects with moderate hypertriglyceridemia are considered to be at increased risk for coronary heart disease (CHD), especially men over age 50 with low high density lipoprotein (HDL) cholesterol levels [1]. Several potential mechanisms have been suggested to contribute to this phenomenon, including an enhanced atherogenic potential of low density lipoprotein (LDL) in the hypertriglyceridemic subjects [2–4]. LDL isolated from hypertriglyceridemic subjects is polydisperse

Abbreviations: CHD, coronary heart disease; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acids; FCH, familial combined hyperlipidemia; HDL, high density lipoprotein; LDL, low density lipoprotein; PUFA, polyunsaturated fatty acids; TG, triglycerides; VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein.

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defined by the presence of multiple LDL subfractions over a broad density range, with the mean LDL subfractions being abnormally small and dense [4,5]. This dense LDL subfraction profile has been associated with an increased risk of CHD [6–8]. In addition, LDL isolated from hypertriglyceridemic subjects is more prone to in vitro oxidative modification than LDL from normotriglyceridemic subjects [4]. The oxidative modification of LDL has been implicated in the initiation and progression of atherosclerosis [9]. So, LDL in hypertriglyceridemic subjects is characterized by a dense LDL subfraction profile and an enhanced susceptibility to oxidation, both contributing to an enhanced atherogenic potential of LDL and thus increased risk of atherosclerosis.

Because of the reported increased risk for premature atherosclerosis, treatment with lipid-lowering drugs is frequently indicated. Both marine n-3 fatty acids (FA) and fibrates are very potent hypotriglyceridemic agents; however, both can also raise LDL cholesterol concentrations, especially in hypertriglyceridemic subjects [10–13]. Only a few studies are available that address the effect of n-3 FA [14–16] and fibrates [4,17] on LDL heterogeneity. Furthermore, dietary n-3 FA are incorporated into lipoproteins, thereby potentially affecting the susceptibility of LDL to oxidative modification. There are conflicting results, however, between studies on the effects of n-3 fatty acid supplementation on LDL oxidizability [16,18–23], whereas only few studies report the effect of fibrates on this parameter [4,17,24].

The present study was undertaken to compare directly the effects of concentrated n-3 FA (Omacor®) vs gemfibrozil on LDL heterogeneity and LDL oxidizability in hypertriglyceridemic patients.

2. Methods

2.1. Patients

A total of 30 patients with primary hypertriglyceridemia (triglyceride (TG) levels between 4.0 and 28.0 mmol/l), confirmed by repeated measurements, were recruited from the outpatient lipid clinic of Nijmegen University Hospital (18 patients) and Amsterdam Academic Medical Centre (12 patients). Exclusion criteria were secondary causes for dyslipidemia, including a history of diabetes mellitus, or apolipoprotein phenotype E2/E2. The participants continued their standard lipid-lowering diet throughout the trial (American Heart Association Step I diet: <30% of total calories/day from fat (maximum 10% saturated fat) and cholesterol <300 mg/day). Other concomitant medication was maintained unchanged during the study. None of the subjects used vitamin supplements, antioxidants or oral blood glucose lowering agents. The protocol was approved by the ethical committee of our institution and written informed consent was obtained from all subjects.

2.2. Study design

This study was a double-blind trial with a double-dummy design. At the start of the study lipid-lowering medication was stopped (week −6), followed by a wash-out period of 4 weeks (week −6 to −2). Baseline plasma lipid values were measured twice at the end of this wash-out period (week −2 and day 0). Thereafter, the subjects were randomly assigned to receive either gemfibrozil (1200 mg/day) together with placebo matching Omacor™ capsules ($n=16$) or Omacor™ capsules (4 g/day) together with placebo matching gemfibrozil for 12 weeks ($n=14$) (day 0 to week 12). Blood samples were obtained at weeks 6, 10 and 12. The Omacor capsules (Pronova Biocare, Oslo, Norway) contained 1 g of concentrated n-3 FA (92%): 44.4% eicosapentaenoic (EPA) and 36.2% docosahexaenoic acid (DHA). D-alpha-tocopherol was added as an antioxidant to a concentration of 4 IU/g = 3.3 mg/g. The placebo capsule contained corn oil (56.3% linoleic acid), mono-unsaturated FA (26.8% oleic acid) and saturated FA (2.3% stearic acid), and 2.4 mg vitamin E.

For the evaluation of adverse events, serum enzyme activities (ALAT and ASAT), glucose and HbA_{1c} were determined according to the clinical routine at the hospital. Compliance was monitored by counting the returned capsules and was 98%.

2.3. Plasma

Venous blood samples were collected after an overnight fast into vacutainer tubes containing 1 mg/ml of ethylenediaminetetraacetic acid (K_3-EDTA). Plasma was isolated immediately and saccharose solution (final concentration 600 mg/ml H_2O) was added to prevent denaturation of lipoproteins during freezing; samples were stored at −80°C. All determinations were performed at the lipid research laboratory of the University Hospital Nijmegen.

2.4. Lipid and lipoprotein analysis

Very low density lipoprotein (VLDL) + intermediate density lipoprotein (IDL) ($d<1.019$ g/ml) were isolated by ultracentrifugation for 16 h at 36 000 rpm (153 000 × g) in a fixed-angle TFT 45.6 rotor (Kontron, Zurich). Plasma and lipoprotein cholesterol and triglyceride concentrations were determined with the Hitachi 744 analyser (cholesterol no 237574; triglyceride no 1361155; Boehringer-Mannheim, FRG). HDL cholesterol was determined in whole plasma using the phosphotungstate/Mg_{2+} method [25]. Apo E phenotypes were determined after iso-electric focusing of

VLDL lipoproteins, as described previously [26]. The apoE phenotypes were E4/3 ($n=10$), E3/3 ($n=6$), E3/2 ($n=12$), and E4/2 ($n=2$).

2.5. Analysis of low density lipoprotein subfraction profiles

LDL subfractions before and after treatment were separated by single spin density gradient ultracentrifugation [27]. Each individual LDL subfraction profile was defined by a continuous variable K, as described in detail previously [28,29]. Briefly, after ultracentrifugation the LDL subfractions were visible as distinct bands in the middle of the tube. Up to five LDL subfractions could be distinguished, i.e. LDL1 ($d=1.030-1.033$ g/ml), LDL2 ($d=1.033-1.040$ g/ml), LDL3 ($d=1.040-1.045$ g/ml), LDL4 ($d=1.045-1.049$ g/ml) and LDL5 ($d=1.049-1.054$ g/ml). The subfractions were carefully aspirated by means of a pasteur pipette. The volumes were calculated by weighing after correction for the densities. Subsequently, cholesterol was determined in each fraction; the concentrations were corrected for dilution and incomplete recoveries. The relative cholesterol concentrations (%chol) in the LDL subfractions were used to calculate parameter K as a continuous variable, which best describes each individual LDL subfraction profile. The relative contribution of each LDL subfraction, expressed by its cholesterol concentration (%chol LDL1–LDL5) relative to the total LDL subfraction profile (total LDL (100%) = %chol LDL1 + %chol LDL2 + %chol LDL3 + %chol LDL4 + %chol LDL5) was calculated. The relative cholesterol concentration of LDL3 and the less frequently occurring LDL4 and/or LDL5 were added to give %chol LDL3′ = (%chol LDL3 + %chol LDL4 + %chol LDL5), where LDL [100%] = LDL1 (%chol LDL1) + LDL2 (%chol LDL2) + LDL3 (%chol LDL3′). When a subfraction pattern was characterized by a predominance of buoyant LDL particles, K was calculated by $K=$ (%chol LDL1 − %chol LDL3′)/(%chol LDL2 − %chol LDL3′) + 1). In the case of a predominancy of heavy, dense LDL particles, K was calculated by $K=$ (%chol LDL1 − %chol LDL3′)/(%chol LDL2 − %chol LDL1) + 1). A negative value ($K<0$) reflects a more dense LDL subfraction profile, and a positive K-value ($K>0$) a more buoyant profile.

2.6. Oxidation of low density lipoproteins

Plasma isolation was immediately followed by LDL isolation by density gradient ultracentrifugation (40 000 rpm for 18 h at 4°C) using a SW40 rotor (Beckman, Palo Alto, CA, USA). After isolation of total LDL, the protein content of LDL was measured by the method of Lowry et al. [30], with chloroform extraction to remove turbidity, using bovine serum albumin as a standard. LDL cholesterol was calculated by subtracting VLDL + IDL cholesterol and HDL cholesterol from total cholesterol. The oxidation experiments were performed as described by Esterbauer et al. [31], as modified by Kleinveld et al. [32]. Briefly, the oxidation of LDL (60 μg apolipoprotein/ml) was initiated by the addition of $CuSO_4$ to a final concentration of 18 μM at 37°C. The kinetics of the oxidation of LDL was determined by monitoring the change of the 234-nm diene absorption in a thermostated UV spectrophotometer. The oxidation characteristics of LDL were determined as described previously by the lag time (min), the oxidation rate (nmol dienes/mg protein per min) and the maximal amount of dienes formed during LDL oxidation (nmol/mg LDL protein) [33].

Thiobarbituric acid reactive substances (TBARS) in plasma were determined as described [34].

2.7. Determination of fatty acids and vitamin E in low density lipoprotein

Analysis of fatty acids, extracted from LDL by vortex mixing with 3 ml n-hexane, was performed by gas chromatography (Varian 3400 GC, Houten, The Netherlands) [33]. Vitamin E concentrations were determined by high-performance liquid chromatography (HPLC Spectra Physics Model 8800), with fluorescence detection. For extraction of vitamin E, 0.2 ml LDL was vortex mixed with 2 ml acetone and 2 ml petroleum ether [35].

2.8. Statistics

The values of the variables measured at week 0 and 12 are presented as the value 'before' and 'after' treatment, respectively. Results are expressed as mean ± S.D. and median with interquartile ranges. The mean of the individual percentage change after therapy was calculated and presented as delta (%).

The effect of either gemfibrozil or Omacor on absolute values of plasma lipoproteins, fatty acid composition, vitamin E concentration, TBARS and LDL oxidizability parameters were tested by non-parametric tests for dependent variables by the Wilcoxon signed rank test. Differences between the effects of gemfibrozil and Omacor on plasma lipoproteins, fatty acid composition, vitamin E concentration, TBARS and LDL oxidizability parameters were tested by the non-parametric Mann–Whitney U-tests for independent variables. A two-tailed probability value of less than 0.05 was considered to be significant. Pearson's correlation coefficients were computed to determine the correlation between the variables fatty acids and oxidizability of LDL. The statistical analyses were performed with procedures available in the SPSS PC + (Statistical Package for the Social Sciences) software package Version 9.0 (SPSS, Chicago, IL, USA).

3. Results

3.1. Patients

Analysis was based on intention-to-treat, but two subjects were not included in the final analysis. One subject (Omacor group) developed excessive hypertriglyceridemia (TG = 56.5 mmol/l) after he stopped his regular medication. One subject (gemfibrozil group) was not willing to continue the trial after randomization.

At baseline, the gemfibrozil group ($n = 13$) and the Omacor group ($n = 15$) were similar in mean age and body mass index (BMI) (mean age 52.7 ± 6.9 vs 48.3 ± 8.3 years, respectively; BMI 26.6 ± 3.8 vs 27.5 ± 2.4 kg/m^2, respectively). After treatment, in the Omacor group eight patients showed an increase in body weight whereas in seven patients body weight remained stable or decreased. Similarly, in the gemfibrozil group six patients showed an increase in body weight whereas seven patients showed a stable or decreased body weight.

After inclusion, in both the gemfibrozil and Omacor group one patient with glucose levels above 6.9 mmol/l was present (gemfibrozil group, $n = 1$, glucose 10.0 and 10.1 mmol/l at week 0 and 12, respectively; Omacor group, $n = 1$, glucose 12.1 and 10.5 mmol/l at week 0 and 12, respectively). All other patients had glucose levels below 7.0 mmol/l. These two patients did not have a history of diabetes mellitus and were not treated with oral blood glucose lowering agents either before or during the trial. The fasting glucose concentrations as well as the hemoglobin A_{1c} concentration were similar at baseline levels for both drugs (gemfibrozil glucose 6.15 ± 2.04 mmol/l and HbA_{1c} 5.66 ± 0.67% vs Omacor glucose 5.88 ± 1.24 mmol/l and HbA_{1c} 5.54 ± 0.55%) and did not change during treatment with Omacor or gemfibrozil (data not shown). Omacor and gemfibrozil were tolerated well by all patients and no significant side-effects were observed.

3.2. The effect of treatment on plasma lipids and lipoprotein levels

The results for lipid and lipoprotein concentrations at baseline and after 12 weeks of treatment with gemfibrozil or Omacor are summarized in Table 1. Although baseline values of triglyceride and cholesterol levels of patients in the Omacor group are higher than in the gemfibrozil group this is not significant (P-value for triglyceride 0.44 and for cholesterol 0.25 (Mann–Whitney U-test)).There were no significant differences between the two groups at baseline. Both gemfibrozil and Omacor significantly reduced total triglyceride levels in plasma as well as in the VLDL fraction. In addition, the VLDL cholesterol significantly decreased in both treatment groups. However, only a slight reduction in total cholesterol was found, reaching statistical significance in the Omacor group only, due to significant increase in HDL cholesterol and LDL cholesterol levels in both groups after treatment. There were no significant differences between the effects of gemfibrozil or Omacor on plasma lipoproteins.

3.3. The effect of treatment on LDL subfraction profile and K-value

The hypertriglyceridemic LDL before therapy tended to be polydisperse, consisting of multiple subfractions (LDL1–LDL5) over a broad density range ($d = 1.030$–1.054 g/ml), with the dense LDL subfractions (LDL3–LDL4) contributing most to total LDL (Fig. 1). This dense LDL subfraction profile is reflected by a negative value for parameter K, which did not differ significantly between the groups at baseline (Table 1). Both gemfibrozil and Omacor increased total LDL cholesterol (Table 1) by increasing the cholesterol content of LDL1, LDL2 and LDL3, whereas the relative contribution of LDL4 and LDL5 to total LDL decreased (Fig. 1), thus resulting in a more buoyant LDL subfraction profile. This is reflected by the increase in the value of parameter K after either gemfibrozil or Omacor therapy (Table 1). The value of parameter K increased more after gemfibrozil (+26.5%, $P < 0.01$) than after Omacor (+10.3%, $P = 0.05$) but the difference in change of parameter K between gemfibrozil and Omacor did not reach statistical significance ($P = 0.088$).

3.4. The effect of treatment on fatty acid composition and vitamin E content of LDL

In the gemfibrozil group the relative contribution of palmitic acid (16:0), and oleic acid (18:1) decreased significantly, whereas that of stearic acid (18:0), linolic acid (18:2), arachidonic acid (20:4), EPA (20:5) and DHA (22:6) did not change significantly (Table 2). In the Omacor group the relative contribution of EPA and DHA increased significantly, with a significant decrease of stearic acid and oleic acid, whereas the relative contribution of linoleic acid and arachidonic acid did not change (Table 2). Vitamin E in LDL increased significantly in both treatment groups. The total amount of polyunsaturated fatty acids (PUFA) in LDL tended to increase in both groups, just reaching statistical significance only in the gemfibrozil group. The ratio PUFA/vitamin E in LDL decreased significantly in both groups (Table 2). No significant difference between the effect of gemfibrozil and Omacor on fatty acid composition and vitamin E content of total LDL was found, except for palmitic acid and DHA and with borderline significance for EPA (Table 2).

3.5. The effect of treatment on oxidation of LDL

The lag time decreased significantly among the subjects treated with Omacor, whereas treatment with gemfibrozil did not affect the lag time (Table 3). A significant difference between the effect of gemfibrozil and Omacor on lag time was found ($P < 0.001$). Although the rate of oxidation tended to increase, the differences were not significant in any treatment group. Total amount of dienes produced per milligram of LDL protein increased in both groups after treatment, reaching statistical significance in the Omacor group and borderline significance in the gemfibrozil group (Table 3). No difference between the effect of gemfibrozil and Omacor on the rate of oxidation and amount of dienes was found (Table 3). TBARS concentrations in plasma increased after treatment with Omacor (+39%) and decreased after treatment with gemfibrozil (−6%); however, both changes failed to reach statistical significance. No significant difference in change in TBARS between both treatment groups was found (Table 3).

4. Discussion

The underlying cause of the increased tendency toward cardiovascular diseases in patients with hypertriglyceridemia is probably related to the enhanced atherogenic potential of their lipoproteins. Possible mechanisms contributing to this increased atherogenicity include the presence of small, dense LDL and the enhanced susceptibility to oxidative modification. In this report we described the baseline lipoprotein concentrations, the LDL subfraction profile and LDL oxidizability of patients with hypertriglyceridemia, and compared the effectiveness of treatment with either gemfibrozil or Omacor on these parameters in a double-blind, double-dummy design.

4.1. Lipids, lipoproteins and LDL heterogeneity

The observed reduction in plasma triglyceride, VLDL cholesterol and VLDL triglyceride concentrations and increase in HDL cholesterol concentrations

Table 1
Changes in lipid and lipoprotein concentration and the LDL subfraction profile (i.e. *K*-values) in subjects with hypertriglyceridemia after treatment with Omacor or gemfibrozil[a]

	Drug	Before	After	Delta (%)	P^b	P^c
Total cholesterol	O	8.85 ± 3.04 7.69 (6.15–11.37)	7.85 ± 2.32 7.16 (6.03–8.14)	−8.9 ± 14.8	<0.05	0.65
	G	7.15 ± 1.60 7.04 (5.76–8.95)	6.47 ± 1.16 6.26 (5.37–7.45)	−7.4 ± 15.3	0.06	
Triglycerides	O	9.79 ± 6.51 6.93 (6.00–11.26)	5.24 ± 2.80 4.53 (3.47–6.50)	−37.1 ± 25.5	<0.001	0.68
	G	6.99 ± 2.93 7.09 (4.63–8.02)	3.58 ± 2.27 2.92 (1.99–4.70)	−40.4 ± 52.6	0.01	
HDL cholesterol	O	0.71 ± 0.17 0.70 (0.56–0.84)	0.77 ± 0.18 0.73 (0.63–0.88)	+11.0 ± 18.5	<0.05	0.29
	G	0.79 ± 0.16 0.84 (0.65–0.93)	0.91 ± 0.19 0.81 (0.77–1.07)	+17.1 ± 21.4	<0.05	
VLDL cholesterol	O	5.17 ± 3.17 4.33 (2.72–9.13)	3.38 ± 2.37 2.53 (1.82–4.11)	−33.3 ± 22.3	<0.001	0.41
	G	3.23 ± 1.38 2.88 (2.36–4.33)	1.58 ± 0.78 1.45 (0.85–2.30)	−39.7 ± 55.2	<0.01	
VLDL triglycerides	O	8.76 ± 5.98 6.06 (5.36–10.54)	4.46 ± 2.31 4.01 (2.90–5.40)	−39.2 ± 26.7	<0.001	0.75
	G	6.22 ± 2.71 6.52 (3.80–7.52)	2.99 ± 2.19 2.27 (1.46–4.23)	−42.2 ± 60.1	0.01	
LDL cholesterol	O	2.97 ± 1.03 2.73 (2.31–3.06)	3.70 ± 1.00 3.65 (2.92–3.99)	+29.7 ± 31.2	0.005	1.00
	G	3.13 ± 0.87 3.65 (2.33–3.86)	3.98 ± 1.12 4.25 (3.28–4.93)	+33.6 ± 49.8	<0.05	
K-value	O	−0.61 ± 0.13 −0.62 (−0.71/−0.55)	−0.55 ± 0.16 −0.57 (−0.64/−0.52)	+10.3 ± 22.4	0.05	0.088
	G	−0.61 ± 0.11 −0.61 (−0.71/−0.52)	−0.45 ± 0.20 −0.41 (−0.60/−0.97)	+26.5 ± 33.4	<0.01	

[a] Values are presented in mmol/l, except parameter *K*. Results are expressed as mean ± S.D. and median with interquartile range. G, gemfibrozil (*n* = 13); HDL, high density lipoprotein; LDL, low density lipoprotein; O, Omacor (*n* = 15); VLDL, very low density lipoprotein. 'Before' are values at week 0. 'After' are values at week 12. Delta (%) is mean of the individual percentage change.
[b] *P*-value for the within treatment group Wilcoxon signed ranks test (before vs after) on absolute values.
[c] *P*-value for the between treatment groups Mann–Whitney *U*-test (gemfibrozil vs Omacor).

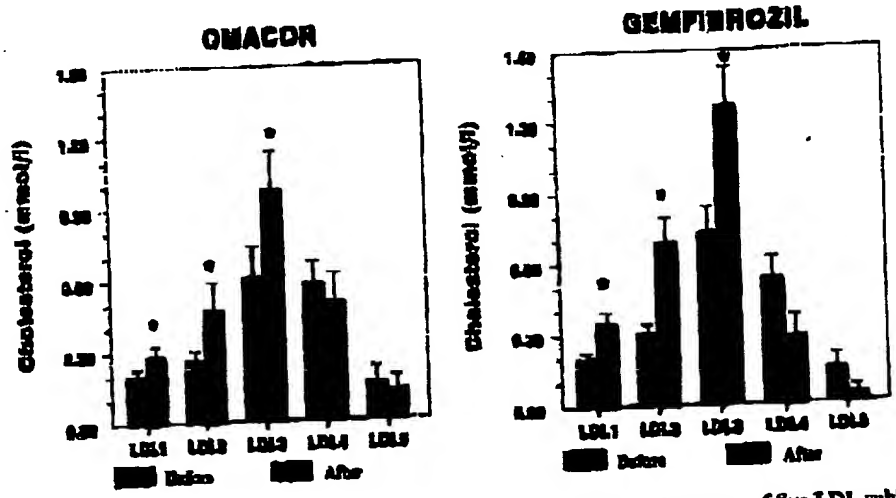

Fig. 1. Effect of treatment with either Omacor ($n=15$) or Gemfibrozil ($n=13$) on the cholesterol content of five LDL subfractions (LDL1–LDL5) of patients with hypertriglyceridemia: 'before' denotes values at week 0; 'after' denotes values at week 12. * P-value <0.05 for the within treatment group. Wilcoxon signed ranks test (before vs after). No significant difference between the effect of Omacor and gemfibrozil treatment on the cholesterol concentration of the LDL subfractions was found.

with gemfibrozil and Omacor after 12 weeks of treatment (Table 1) are in accordance with previous reports [10–12,36]. Our knowledge of the possible mechanisms by which fibrates and eicosanoids induce these changes in lipid and lipoprotein concentrations has evolved greatly since the identification of the peroxisome proliferator-activated receptors (PPARs) [37]. Both eicosanoids and fibrates activate PPARs resulting in an enhanced catabolism of triglyceride-rich particles by decreased production of apoCIII and induction of LPL gene expression [38]. In addition, PPAR activation results in reduced secretion of VLDL particles by increased beta oxidation of fatty acids and inhibition of de-novo fatty acid synthesis [39]. The increase in LDL cholesterol by ~30% on both therapies is slightly larger than previously reported [10–12,36]. The depletion of triglycerides in the VLDL+IDL fraction induced by gemfibrozil and Omacor, leading to small, more dense VLDL+IDL particles which are more likely to be converted into LDL particles, has been suggested to be a cause of the observed increase in LDL cholesterol. So, in this study the high triglyceride levels at baseline may have contributed to the rather marked increase in LDL cholesterol concentrations [40].

The main LDL subfractions before therapy were abnormally small and dense (LDL3 and LDL4), resulting in a dense LDL subfraction profile, reflected by a negative value of parameter K. A dense LDL subfraction profile has been associated with an increased risk for CHD [6–8]. The predominance of small dense LDL in hypertriglyceridemia can be explained by exchange of LDL cholesteryl ester for VLDL triglyceride, mediated by cholesteryl ester transfer protein, followed by subsequent action of lipoprotein lipase or hepatic lipase, resulting in hydrolysis of LDL triglycerides and thereby decreasing LDL particle size [41]. Both gemfibrozil and Omacor treatment resulted in a more buoyant LDL subfraction profile, reflected by the increase in the value of parameter K (Fig. 1 and Table 1). So, Omacor and gemfibrozil adversely raise LDL cholesterol concentration but the increase in LDL cholesterol concentration reflects a less atherogenic light LDL subfraction profile that may be favorable. Similar results have been reported in hypertensive subjects [14] and in patients with familial combined hyperlipidemia (FCH) [15] after Omacor treatment and in hypertriglyceridemic subjects [4] and in patients with FCH [17] after clofibrate and gemfibrozil treatment, respectively. However, Omacor treatment in normolipidemic healthy subjects decreased LDL lipids and increased LDL apoB, thus decreasing the cholesterol/apoB ratio, reflecting more dense LDL, whereas no detectable differences in LDL size was found [16]. A possible explanation for this contrasting result is that the change in LDL composition depends on the extent of triglyceride transfer and lipolysis, determined by the degree of hypertriglyceridemia, which differed between the different reports.

Reportedly, alterations in composition of LDL particles were associated with changes in LDL metabolism in cultured cells, which may render them more atherogenic [2,3]. Another potential mechanism that increases the atherogenicity of LDL includes the oxidative modification [9].

4.2. LDL oxidizability

Oxidative modification of LDL involves the peroxidation of unsaturated fatty acids found within the LDL phospholipid monolayer. Several studies have shown that various types of fatty acids can alter LDL particle susceptibility to oxidative modification [42–44].

The results of studies on the effects of n-3 FA on LDL oxidizability are contradictory. In some of the studies enhanced peroxidation of LDL was observed [18–20], whereas other studies showed no effect of dietary n-3 FA on LDL oxidation [16,21,22]. Different experimental conditions among studies, e.g. in duration of supplementation period, type of patients included, amount of n-3 FA provided, may explain some of the apparently conflicting results obtained regarding the effects of n-3 FA on LDL oxidation.

We show that the lag time of LDL oxidation was significantly shortened by Omacor, which indicates an increase in the susceptibility to oxidation of LDL, as reported previously [18–20]. The trend of increase in TBARS concentration in plasma after treatment with Omacor corresponds with the increased susceptibility to oxidation of LDL in vitro. Several studies have demonstrated that small, dense LDL is more prone to oxidative modification in vitro than the large, light LDL, as measured by the decreased lag time, preceding the onset of the lipid peroxidation, suggesting an enhanced atherogenic potential of the small dense LDL subfrac-

Table 2
Change in fatty acid composition and vitamin E content of total LDL after treatment with Omacor or gemfibrozil in subjects with hypertriglyceridemia[a]

	Drug	Before	After	Delta (%)	P^b	P^c
Palmitic acid (C16:0)	O	23.3 ± 2.0 23.6 (20.8–25.0)	23.6 ± 2.2 23.8 (21.6–25.4)	+1.6 ± 6.6	0.39	0.022
	G	24.1 ± 2.0 23.6 (23.0–25.5)	23.1 ± 3.0 23.0 (22.0–23.6)	−4.0 ± 5.7	0.05	
Stearic acid (C18:0)	O	7.9 ± 0.7 7.9 (7.7–8.5)	7.5 ± 0.6 7.3 (6.8–8.2)	−5.1 ± 7.8	0.015	0.65
	G	7.5 ± 0.5 7.3 (7.2–7.7)	7.1 ± 0.7 7.0 (6.5–7.6)	−4.8 ± 11.0	0.14	
Oleic acid (C18:1 n-9)	O	19.5 ± 2.2 20.1 (17.6–21.0)	17.6 ± 1.6 17.3 (16.4–18.9)	−9.2 ± 8.4	0.003	0.47
	G	18.6 ± 2.8 19.6 (16.1–20.6)	17.7 ± 4.2 18.5 (14.3–19.3)	−5.0 ± 14.8	0.02	
Linoleic acid (C18:2 n-6)	O	40.8 ± 3.5 41.1 (37.9–41.5)	39.8 ± 5.3 39.5 (36.0–45.2)	−2.2 ± 10.7	0.31	0.16
	G	40.8 ± 5.3 41.3 (36.7–45.6)	42.1 ± 5.3 43.1 (36.7–47.8)	+3.3 ± 11.1	0.15	
Arachidonic acid (C20:4 n-6)	O	5.9 ± 1.2 5.9 (4.9–6.6)	5.8 ± 1.4 5.2 (5.0–6.5)	−1.0 ± 14.5	0.73	0.93
	G	7.0 ± 1.4 6.7 (6.0–8.2)	7.0 ± 1.6 7.4 (5.3–7.6)	+0.1 ± 15.3	0.92	
EPA (C20:5 n-3)	O	1.0 ± 0.5 1.1 (0.6–1.3)	3.1 ± 1.9 3.6 (0.8–4.9)	+231.0 ± 238.3	0.005	0.065
	G	0.9 ± 0.7 0.7 (0.5–1.5)	1.5 ± 1.4 0.8 (0.6–2.7)	+82.6 ± 183.6	0.22	
DHA (C22:6 n-3)	O	1.7 ± 0.5 1.7 (1.3–2.0)	2.6 ± 0.8 2.9 (1.8–3.1)	+68.7 ± 66.2	0.006	0.046
	G	1.3 ± 0.6 1.2 (0.8–1.7)	1.6 ± 0.8 1.5 (0.8–2.4)	+20.2 ± 58.4	0.42	
Vitamin E (μmol/g LDL protein)	O	6.53 ± 1.71 7.10 (4.60–7.90)	8.41 ± 1.97 8.30 (7.10–10.1)	+36.5 ± 50.0	<0.05	0.89
	G	6.95 ± 2.39 7.10 (5.05–8.50)	8.87 ± 3.11 8.40 (6.25–10.05)	+37.6 ± 59.4	<0.05	
Total amount of PUFA in LDL (μmol)	O	3596 ± 326 3513 (3323–3963)	3779 ± 535 3752 (3341–4251)	+5.0 ± 10.8	0.09	0.92
	G	3839 ± 271 3872 (3702–4048)	4008 ± 398 4000 (3758–4241)	+4.5 ± 7.0	<0.05	
PUFA/vitamin E in LDL (μmol/mg)	O	592 ± 183 523 (461–738)	468 ± 103 443 (372–590)	−17.4 ± 18.5	<0.001	0.79
	G	628 ± 261 529 (446–764)	499 ± 161 467 (407–652)	−14.8 ± 26.5	<0.001	

[a] Values of fatty acids are presented in percentage of total fatty acids as mean ± S.D. and median with interquartile ranges. DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; G, gemfibrozil ($n = 13$); LDL, low density lipoprotein; O, Omacor ($n = 15$); PUFA, polyunsaturated fatty acids. 'Before' are values at week 0. 'After' are values at week 12. Delta (%) is mean of the individual percentage change.
[b] P-value for the within treatment group Wilcoxon signed ranks test (before vs after) on absolute values.
[c] P-value for the between treatment groups Mann–Whitney U-test (gemfibrozil vs Omacor).

Table 3
Change in LDL oxidizability after treatment with Omacor or gemfibrozil[a]

	Drug	Before	After	Delta (%)	P^b	P^c
Lag time	O	85.7 ± 8.28 85.6 (80.5–92.0)	69.7 ± 8.2 68.0 (66.2–74.5)	−18.6 ± 7.6	<0.001	0.001
	G	74.6 ± 8.8 72.9 (69.5–81.2)	75.3 ± 10.0 72.7 (67.3–85.6)	+2.3 ± 13.8	0.70	
Oxidation rate	O	11.0 ± 1.83 10.6 (9.9–11.9)	11.2 ± 2.65 10.9 (8.1–13.2)	+2.0 ± 20.7	0.84	0.34
	G	11.9 ± 1.60 12.0 (11.1–12.9)	12.9 ± 2.63 12.8 (11.3–15.0)	+10.4 ± 23.4	0.14	
Dienes	O	468 ± 54 443 (431–494)	522 ± 90 521 (438–592)	+14.0 ± 17.0	0.01	0.62
	G	499 ± 46 498 (462–534)	541 ± 81 532 (507–603)	+9.9 ± 13.3	0.055	
TBARS	O	1.48 ± 0.74 1.19 (0.97–1.65)	1.88 ± 0.86 1.73 (1.17–2.54)	+38.7 ± 65.7	0.14	0.16
	G	1.24 ± 0.19 1.05 (0.93–1.58)	1.04 ± 0.19 1.06 (0.90–1.20)	−5.8 ± 36.2	0.34	

[a] Values are presented as mean ± S.D. and median with interquartile ranges; lag time in minutes; oxidation rate in nmol dienes/mg protein per min; dienes in nmol/mg LDL protein. G, gemfibrozil ($n = 13$); O, Omacor ($n = 15$); TBARS, thiobarbituric acid reactive substances (O: $n = 10$; G: $n = 9$). 'Before' are values at week 0. 'After' are values at week 12. Delta (%) is mean of the individual percentage change.
[b] P-value for the within treatment group Wilcoxon signed ranks test (before vs after) on absolute values.
[c] P-value for the between treatment groups Mann–Whitney U-test (gemfibrozil vs Omacor).

tions within each LDL subfraction profile [4,32]. In contrast, we now report that Omacor treatment is associated with a more buoyant LDL subfraction profile and an enhanced susceptibility to oxidation of total LDL. Since the ratio total PUFA per vitamin E in LDL decreased after Omacor, we may assume that the increased n-3 FA content most likely caused the increased susceptibility to oxidation, as the degree of unsaturation of fatty acids is one of the main determinants of the susceptibility of the lipoproteins to oxidation.

After gemfibrozil treatment the lag time and TBARS concentration did not change significantly. To our knowledge only three reports have previously published the effect of fibrates on LDL oxidizability in humans [4,17,24]. In primary hypertriglyceridemic subjects, clofibrate treatment reduced the susceptibility of LDL to oxidation, as measured by a significant increase in lagtime in isolated LDL subfractions after therapy [4]. In subjects with familial combined hyperlipidemia gemfibrozil also tended to increase the resistence of total LDL to oxidation, as the lag time increased after therapy, although not reaching statistical significance [17]. In patients with hyperlipidemia type IIA and IIB, bezafibrate reduced the propensity of LDL to undergo lipid peroxidation in vitro [24]. The mechanism by which fibrates exhibit antioxidant potential is still unknown. One study reports that the p-hydroxy-metabolite I is involved through free radical scavenger activity [45]. Our present data show only little effect on LDL oxidizability after gemfibrozil treatment, less than expected on the basis of the more buoyant LDL subfraction profile induced by gemfibrozil. A possible explanation is that LDL oxidizability is determined in total LDL, which is the addition of maximal five LDL subfractions, so small changes might remain undetected.

The rate of LDL oxidation did not change in either group. The maximal amount of dienes formed per milligram of LDL protein during oxidation of LDL was significantly increased after Omacor therapy. This could be attributed to the increased number of oxidizable groups (=double bands) in LDL due to Omacor supplementation. Indeed, a significant correlation between the PUFA content and diene production was found in the Omacor group ($r = 0.61$, $P < 0.01$).

5. Conclusion

Gemfibrozil and Omacor have anti-atherogenic properties, as both therapies reduce the atherogenic potential of the lipoproteins by decreasing the concentration of cholesterol-enriched VLDL and increasing HDL concentration. Although total plasma LDL cholesterol concentration increases, the atherogenic potential of LDL seems to be less, as judged by the presence of a more buoyant LDL subfraction profile. In contrast to gemfibrozil, Omacor increased the susceptibility of LDL to oxidation in vitro. Although this could be unfavorable, it does not necessarily mean that n-3 FA are atherogenic in vivo. In animal studies, the incorporation of n-3 FA into LDL particles rendered them more susceptible to oxidation in vitro, but no increase

in atherosclerotic lesion development [46] or even an anti-atherogenic effect [47] in vivo was found. These findings might be explained by the presence in vivo of antioxidant mechanisms that can attenuate the increased potential of n-3 FA in LDL to undergo oxidative modification. Furthermore, n-3 FAs are reported to have a wide range of biological effects that may be related to protection against atherogenesis, i.e. reduction of platelet aggregation and vasoconstriction [48–52] and antiarrhythmic effects [53]. These mechanisms can to some degree offset the potential unfavorable effect of n-3 FA incorporation into LDL. Alternatively, supplementation of n-3 FA with anti-oxidants may help prevent the susceptibility of LDL to peroxidative modification.

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A Nutriti nal Supplement For Lowering Serum Triglyceride and Cholesterol Levels

Field of the Invention

The invention relates to control of cholesterol and triglyceride levels in mammals, particularly humans.

Background of the Invention

Serum cholesterol and serum triglyceride levels are important factors in the development of cardiovascular disease. In many clinical studies there is a positive correlation between plasma triglycerides and the incidence of cardiovascular disease [1]. Elevated plasma triglyceride level is frequently associated with other atherogenic factors including elevated low-density lipoprotein (LDL)-cholesterol, reduced high-density lipoprotein (HDL)-cholesterol, and small LDL particles [2, 3]. There is growing acceptance that triglycerides act in a synergistic fashion with these other lipid risk factors to increase the incidence of cardiovascular disease [4, 5]. Hypertriglyceridemia usually occurs because of insulin resistance, which leads to overproduction of very low-density lipoproteins (VLDL) by the liver [3]. Treatment involves lifestyle changes to decrease body weight and to increase physical activity, both of which improve insulin sensitivity. Drug therapy to lower triglycerides involves the use of fibrates or nicotinic acid [6].

A number of clinical studies convincingly establish plasma cholesterol and LDL-cholesterol as independent risk factors for coronary heart disease [7]. Pharmacological agents, called statins, lower total plasma cholesterol by inhibiting the synthesis of cholesterol by the liver. The

statins reduce the morbidity and mortality rate from cardiovascular disease in high risk, hypercholesterolemic patients [8, 9], but also in persons who exhibit "average" cholesterol levels [10]. Another approach is to interfere with the intestinal absorption of cholesterol. Certain phytosterols (plant sterols) such as stigmasterol and β-sitosterol lower serum cholesterol act by inhibiting absorption of both dietary and biliary cholesterol from the small intestine [11].

With respect to the most appropriate form of phytosterols for lowering serum cholesterol, some reports indicate that free phytosterols reduce serum cholesterol in animals and humans [12, 13]. However, there is also evidence to indicate that a sterol esterified with a fatty acid may be more effective [14]. Trials show that phytosterol esters of plant fatty acids obtained from canola oil, when incorporated into food such as margarine or mayonnaise, lower total cholesterol and LDL-cholesterol levels by about 10 and 15 percent, respectively [15, 16]. United States Patent No. 5,502,045 (Miettinen et al., issued March 26, 1996) discloses the use of sitostanol esters of canola oil to lower serum cholesterol. Benecol™ (Raisio Benecol Ltd., Raisio, Finland), a margarine that contains such compounds, is now on the market.

The mechanism by which phytosterols or phytosterol esters inhibit absorption of dietary cholesterol by the digestive tract is not fully understood but may involve competitive inhibition of cholesterol uptake from the intestinal lumen or inhibition of cholesterol esterification in the intestinal mucosa [12]. It is known that phytosterols themselves are only poorly absorbed. Vanhanen et al. [17] report that phytosterol esters may also be poorly absorbed by

the intestinal tract based on postprandial measurements of β-sitostanol in plasma. A direct measure of phytosterol ester uptake by the digestive tract has not been reported.

When phytosterols are esterified with fatty acids from plant sources such as canola, the long-chain polyunsaturated fatty acids (LCPUFAs) that are incorporated are predominantly of the omega-6 series. Omega-6 fatty acids do not affect plasma triglycerides. Research to date on fatty acid esters of sterols has focused only on the efficacy of the sterol in lowering cholesterol.

Summary of the Invention

The present invention provides a nutritional supplement comprising a sterol and an omega-3 fatty acid, or an ester thereof, for lowering cholesterol and triglyceride levels in the bloodstream of a subject.

The present invention also provides a method of lowering cholesterol and triglyceride levels in the bloodstream of a subject, the method including the step of administration of an effective amount of a nutritional supplement comprising a sterol and an omega-3 fatty acid, or an ester thereof, to a subject.

The present invention also provides the use of the nutritional supplement defined herein for lowering cholesterol and triglyceride levels in the bloodstream of a subject.

The subject is preferably a mammal, more preferably a human.

The present invention further provides a foodstuff composition comprising the nutritional supplement defined

herein and a foodstuff, the nutritional value of the foodstuff being enhanced by incorporation of the nutritional supplement defined herein.

The present invention further provides the use of the nutritional supplement defined herein in the manufacture of a foodstuff composition.

The present invention further provides a process for preparing the nutritional supplement as defined herein, which comprises the step of reacting a sterol with an omega-3 fatty acid, or an ester thereof, in the presence of a base.

Base catalysts were found to be successful in the transesterification (or interesterification) process of the invention. Such a reaction is advantageous given the availability of esterified omega-3 fatty acid starting material, for example from fish oil. In addition, acidic catalysts were found to be ineffective in the transesterification of interest.

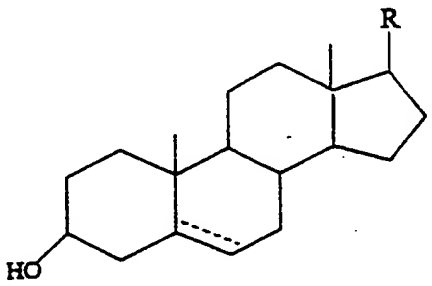
Sterols are not very soluble in lipid, which complicates their use in lipid-based foods. A mixture of a sterol and a free omega-3 fatty acid, which typically forms a paste at a molar ratio of 1:1, may be used. If a mixture is used, the omega-3 fatty acid can be a free acid or can be in ester form, preferably a succinimidyl, triglyceride, (C_3-C_{12})cycloalkyl or (C_1-C_8)alkyl ester, more preferably an ethyl ester. In the mixture, the molar ratio range of omega-3 fatty acid, or an ester thereof, to sterol should be about 0.5 to 8 , preferably 0.76 to 6.4, more preferably 1 to 2.

Preferably, the sterol and the omega-3 fatty acid are together in the form of an ester. The sterol esters of the

present invention are highly fat-soluble and represent a bifunctional species, since they lower both serum cholesterol and serum triglyceride levels in the bloodstream.

Detailed Description of the Preferred Embodiments

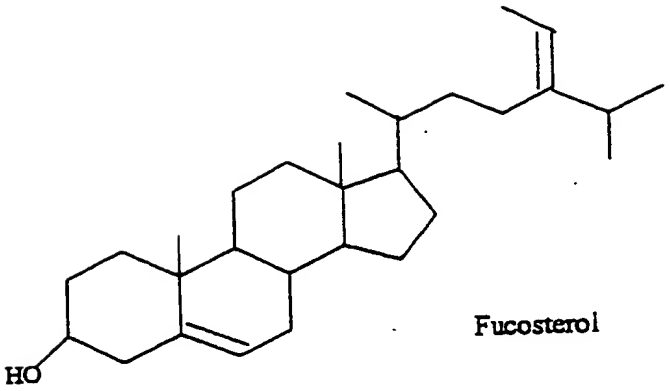
The sterols used to prepare the nutritional supplement of the present invention are preferably phytosterols, and preferably have a perhydrocyclopentanophenanthrene ring system as shown below in the compound of formula I:



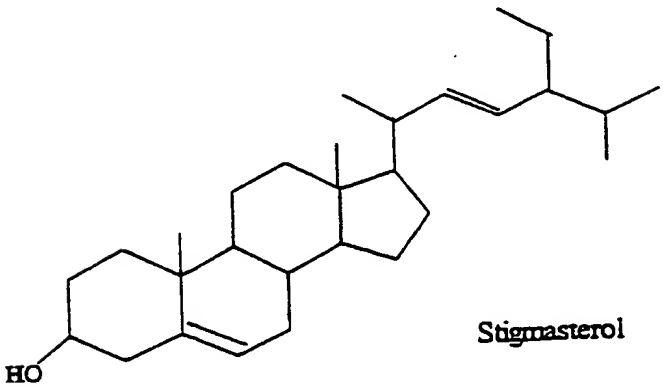
(I)

wherein the dashed line is a single or double bond and R is a (C_1-C_{10})alkyl, substituted (C_1-C_{10})alkyl, (C_2-C_{10})alkenyl or substituted (C_2-C_{10})alkenyl group.

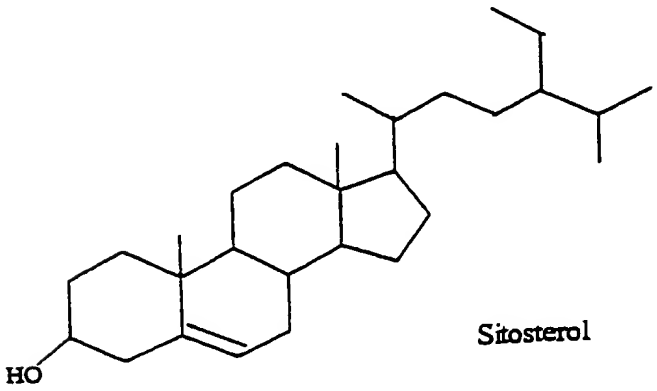
In the present application, the term "sterols" includes sterols in reduced form (stanols), preferably β-sitostanol or fucostanol (reduced fucosterol).



Fucosterol



Stigmasterol

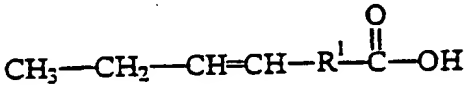


Sitosterol

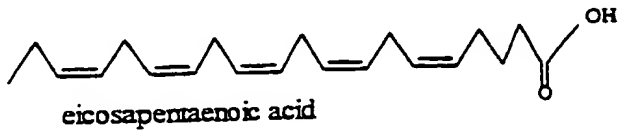
One or more sterols can be used to prepare the nutritional supplement. The term "phytosterols" includes sterols from terrestrial or marine plants, seaweed, microalgae, etc. Preferably, the sterol is stigmasterol, sitosterol, fucosterol, β-sitostanol or fucostanol.

Fucosterol is abundant in brown algae. Prior to esterification with the omega-3 fatty acid, fucosterol can be reduced to fucostanol. Preferably, the reduction is carried out using hydrogen gas in the presence of a suitable catalyst such as palladium on charcoal (Pd/C), but other reduction processes that ultimately yield a food-quality ester, after purification if necessary, may be used.

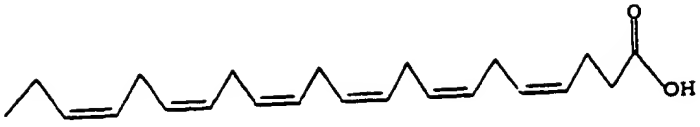
The nutritional supplement of the present invention comprises one or more omega-3 fatty acids, and is preferably an ester of an acid of the formula:



wherein R^1 is a (C_3-C_{40})alkenylene group comprising at least one double bond, more preferably 2 to 5 double bonds. More preferably, the omega-3 fatty acid is stearidonic acid 18:4ω3 (SA), eicosapentaenoic acid 20:5ω3 (EPA) or docosahexaenoic acid 22:6ω3 (DHA).



eicosapentaenoic acid



docosahexaenoic acid

Omega-3 fatty acids, such as EPA and DHA, are long-chain polyunsaturated fatty acids (LCPUFAs) that are abundant in oily fish such as menhaden, salmon, tuna, and sardine, as well as in certain plants and microbes, such as particular fungi and microalgae. The preferred source of omega-3 fatty acids for the present invention is fish oil, more preferably a highly refined fish oil concentrate having approximately 65% omega-3 fatty acid content which is predominantly EPA and DHA in the form of triglyceride esters. These triglycerides are preferably converted to lower alkyl esters, such as methyl, ethyl or propyl esters, by known methods and used in an esterification with a sterol to form esters, which can be further purified if necessary, for use as nutritional supplements.

The cardiovascular effects of dietary fish oils have long been recognized [18, 19]. Omega-3 fatty acids lower plasma triglyceride concentrations principally by inhibiting synthesis of triacylglycerol and VLDL by the liver [20]. In addition, omega-3 fatty acids are anti-thrombotic and are protective against cardiac arrhythmias [21]. The benefits of fish oil consumption are illustrated by the finding of the Diet and Reinfarction Trial (DART) which showed a reduction of 29% in the overall mortality in survivors of a first myocardial infarction who consumed fish rich in omega-3 fatty acids at least twice weekly [22]. Two recent studies demonstrate the

efficacy of omega-3 fatty acid supplementation. In a randomized, double-blind, placebo-controlled trial patients with coronary artery disease who ingested a 1.5g/day fish oil supplement (55% EPA and DHA) for two years had less progression and more regression of their disease based on coronary angiography compared to patients ingesting the placebo [23]. In the GISSI- Prevenzione trial, omega-3 fatty acid supplements in patients who had myocardial infarction reduced cardiovascular death by 30% [24]. Although omega-3 fatty acids are anti-atherogenic, they do not lower plasma cholesterol and in some incidences may slightly increase LDL-cholesterol [25]. Safety and toxicological studies spanning several years have shown that fish oils are safe to consume. Recently, fatty acids such as the omega-3 fatty acids from fish oil were granted GRAS (Generally Regarded As Safe) status in the United States, which permits their addition to foods low in long-chain polyunsaturated fatty acids. The typical North American diet contains about 0.15 grams omega-3 fatty acids whereas Inuit may ingest up to 10 grams of omega-3 fatty acids daily. A daily intake of 2 to 3 grams of omega-3 fatty acids has consistently been shown to lower plasma triglycerides [18]. Therefore, a suitable daily intake of omega-3 fatty acid in the present invention is about 0.1 to about 10 grams, preferably about 2 to about 3 grams, but clearly greater amounts can be tolerated, and may be beneficial.

Phytosterols are considered safe for human consumption. A typical daily intake in North America is about 100 to 300 milligrams. However, a dose of greater than 3 grams of the phytosterol esters are required to have significant impact on plasma cholesterol levels [13]. Such doses are safe with no known side effects. In the present invention, a

preferred daily intake of phytosterol is about 2 to about 3 grams.

Phytosterol esters prepared using fish oil as the source of omega-3 fatty acids contain a significant amount of EPA and DHA. Such esters can simultaneously reduce serum cholesterol and serum triglyceride levels. The triglyceride-lowering ability of the omega-3 fatty acid component of the ester is dependent on its entry into the circulatory system. A lipid esterase in the intestinal lumen may be responsible for release of the omega-3 fatty acid from the phytosterol, which would make both species available for uptake into the circulatory system. There is a non-specific lipid esterase, secreted into the intestinal lumen during digestion that is active against a variety of molecular species including cholesterol esters, monoglycerides, and esters of vitamin A [26].

At least one edible additive, such as listed below, can be included for consumption with the nutritional supplement of the invention and may have, for example, antioxidant, dispersant, antimicrobial, or solubilizing properties. A suitable antioxidant is, for example, vitamin C, vitamin E or rosemary extract. A suitable dispersant is, for example, lecithin, an alkyl polyglycoside, polysorbate 80 or sodium lauryl sulfate. A suitable antimicrobial is, for example, sodium sulfite or sodium benzoate. A suitable solubilizing agent is, for example, a vegetable oil such as sunflower oil, coconut oil, and the like, or mono-, di- or tri-glycerides.

Additives include vitamins such as vitamin A (retinol, retinyl palmitate or retinol acetate), vitamin B1 (thiamin, thiamin hydrochloride or thiamin mononitrate),

vitamin B2 (riboflavin), vitamin B3 (niacin, nicotinic acid or niacinamide), vitamin B5 (pantothenic acid, calcium pantothenate, d-panthenol or d-calcium pantothenate), vitamin B6 (pyridoxine, pyridoxal, pyridoxamine or pyridoxine hydrochloride), vitamin B12 (cobalamin or cyanocobalamin), folic acid, folate, folacin, vitamin H (biotin), vitamin C (ascorbic acid, sodium ascorbate, calcium ascorbate or ascorbyl palmitate), vitamin D (cholecalciferol, calciferol or ergocalciferol), vitamin E (d-alpha-tocopherol, d-beta-tocopherol, d-gamma-tocopherol, d-delta-tocopherol or d-alpha-tocopheryl acetate) and vitamin K (phylloquinone or phytonadione).

Other additives include minerals such as boron (sodium tetraborate decahydrate), calcium (calcium carbonate, calcium caseinate, calcium citrate, calcium gluconate, calcium lactate, calcium phosphate, dibasic calcium phosphate or tribasic calcium phosphate), chromium (GTF chromium from yeast, chromium acetate, chromium chloride, chromium trichloride and chromium picolinate) copper (copper gluconate or copper sulfate), fluorine (fluoride and calcium fluoride), iodine (potassium iodide), iron (ferrous fumarate, ferrous gluconate or ferrous sulfate), magnesium (magnesium carbonate, magnesium gluconate, magnesium hydroxide or magnesium oxide), manganese (manganese gluconate and manganese sulfate), molybdenum (sodium molybdate), phosphorus (dibasic calcium phosphate, sodium phosphate), potassium (potassium aspartate, potassium citrate, potassium chloride or potassium gluconate), selenium (sodium selenite or selenium from yeast), silicon (sodium metasilicate), sodium (sodium chloride), strontium, vanadium (vanadium sulfate) and zinc (zinc acetate, zinc citrate, zinc gluconate or zinc sulfate).

Other additives include amino acids, peptides, and related molecules such as alanine, arginine, asparagine, aspartic acid, carnitine, citrulline, cysteine, cystine, dimethylglycine, gamma-aminobutyric acid, glutamic acid, glutamine, glutathione, glycine, histidine, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine, taurine, threonine, tryptophan, tyrosine and valine.

Other additives include animal extracts such as cod liver oil, marine lipids, shark cartilage, oyster shell, bee pollen and d-glucosamine sulfate.

Other additives include unsaturated free fatty acids such as γ-linoleic, arachidonic and α-linolenic acid, which may be in an ester (e.g. ethyl ester or triglyceride) form.

Other additives include herbs and plant extracts such as kelp, pectin, Spirulina, fiber, lecithin, wheat germ oil, safflower seed oil, flax seed, evening primrose, borage oil, blackcurrant, pumpkin seed oil, grape extract, grape seed extract, bark extract, pine bark extract, French maritime pine bark extract, muira puama extract, fennel seed extract, dong quai extract, chaste tree berry extract, alfalfa, saw palmetto berry extract, green tea extracts, angelica, catnip, cayenne, comfrey, garlic, ginger, ginseng, goldenseal, juniper berries, licorice, clive oil, parsley, peppermint, rosemary extract, valerian, white willow, yellow dock and yerba mate.

Other additives include enzymes such as amylase, protease, lipase and papain as well as miscellaneous substances such as menaquinone, choline (choline bitartrate), inositol, carotenoids (beta-carotene, alpha-carotene, zeaxanthin, cryptoxanthin or lutein), para-aminobenzoic acid, betaine HCl,

free omega-3 fatty acids and their esters, thiotic acid (alpha-lipoic acid), 1,2-dithiolane-3-pentanoic acid, 1,2-dithiolane-3-valeric acid, alkyl polyglycosides, polysorbate 80, sodium lauryl sulfate, flavanoids, flavanones, flavones, flavonols, isoflavones, proanthocyanidins, oligomeric proanthocyanidins, vitamin A aldehyde, a mixture of the components of vitamin A_2, the D Vitamins (D_1, D_2, D_3 and D_4) which can be treated as a mixture, ascorbyl palmitate and vitamin K_2.

The nutritional supplement of the invention is typically a viscous oil and can be added to a foodstuff composition during processing of the foodstuff. Such a foodstuff composition is often referred to as a functional food, and can be any food that will tolerate the physicochemical properties of the nutritional supplement, for example, margarine, cooking oil, shortening or mayonnaise. It can also be packaged for consumption in softgel, capsule, tablet or liquid form. It can be supplied in edible polysaccharide gums, for example carrageenan, locust bean gum, guar, tragacanth, cellulose and carboxymethylcellulose.

The nutritional supplement can also be microencapsulated. Microencapsulation can be carried out, for example, using a gelatin such as bovine gelatin in a co-extrusion process, prior to processing into a foodstuff composition, for example baked goods, candy, margarines and spreads, ice cream, yogurts, frozen desserts, cake mixes and pudding mixes. The packaging of the nutritional supplement should preferably provide physical protection from such effects as pH, particularly basic conditions, oxidation and degradation by light. This latter effect can be minimized for example by changing the mesh size of the microencapsulation or inclusion

of a suitable dye. The nutritional supplement can also be stored in a light-opaque container to minimize photodegradation.

The example below describes synthesis of an ester of the invention. The ester linkage can be formed according to known methods, such as by esterification of free fatty acids by sterols or stanols under acid catalysis (US Patent No. 5,892,068: Higgins III, issued April 6, 1999). Preferably, however, a base is used as a catalyst to promote transesterification. More preferably, the base is a metal (C_1-C_{10})alkoxide, even more preferably sodium methoxide or ethoxide. Conveniently, the reactants are heated to a temperature of about 100°C to about 200°C with stirring, preferably under reduced pressure, for about 30 minutes to about 4 hours. The base is then added and the mixture conveniently stirred at a temperature of about 100°C to about 200°C under reduced pressure for about 30 minutes to about 36 hours. Alternatively, the starting ester is heated to a temperature of about 100°C to about 200°C with stirring, preferably under reduced pressure, for about 30 minutes to about 4 hours. The base dispersed in the phytosterol is then added and the mixture conveniently stirred at a temperature of about 100°C to about 200°C under reduced pressure for about 30 minutes to about 36 hours. The ester that is formed can be further purified if necessary for use as a nutritional supplement.

The further purification is preferably carried out by precipitation and extraction, preferably sequentially, using two immiscible solvents. Unreacted sterol is precipitated by addition of a suitable non-polar solvent and filtered off. A suitable non-polar solvent can be an aliphatic liquid such as a

liquid alkane, preferably pentane, hexane, heptane, octane, isooctane or dodesane, more preferably hexane. Corresponding fluoroalkanes can also be used. The non-polar solvent can also be an aromatic solvent such as benzene or toluene, or an other solvent of similar polarity such as carbon tetrachloride or methyl-tert-butyl ether.

The filtrate is then extracted by a suitable extraction solvent to remove unreacted omega-3 fatty acid-containing material. The extraction solvent is preferably a polar solvent such as methanol, ethanol or ethylene glycol dimethyl ether (monoglyme), more preferably methanol. Certain dipolar aprotic solvents, such as N,N-dimethyl formamide (DMF) or dimethylsulfoxide (DMSO), can also be used.

Example 1

Synthesis of Stigmasterol/Omega-3 Fatty Acid Esters.

(A) A mixture of dry stigmasterol (3 g, 7.27 mmol) and a highly concentrated mixture of EPA and DHA omega-3 fatty acids in ethyl ester form (EPAX™ 5500, ProNova; 4.3 g, 12.6 mmol) were heated while being stirred magnetically at 140 to 145°C for 2 hours under vacuum (5 mm). Subsequently the vacuum was disconnected and powdered sodium methoxide (40 mg, 0.75 mmol) was added quickly in one portion. The vacuum was connected immediately and the mixture was stirred at 140 to 145°C for an additional 4 hours. Hexane (25 mL) was added to precipitate the residual stigmasterol and the mixture was centrifuged for 5 minutes at 15,000 g (0°C), the supernatant was removed and the pellet was washed again with 5 mL of hexane. The remaining precipitate was centrifuged off and the supernatants combined. The organic phase was washed with water

(5 mL), dried over sodium sulfate and the solvent removed under reduced pressure. TLC (hexane/diethylether/acetic acid (90:10: 1), R_f 0.71. The yield was 5.9 g (85%). The ester product was a viscous oil.

When the experiment was repeated using freshly made sodium ethoxide, almost the same level of conversion was obtained as with sodium methoxide. However, this was not seen with commercially available sodium ethoxide, which performed more poorly than sodium methoxide.

Synthesis of Stigmasterol/Omega-3 Fatty Acid Esters

(B) A highly concentrated mixture of EPA and DHA omega-3 fatty acids in ethyl ester form (EPAX™ 5500 EE, BioNova; 221 g, 649 mmol) was heated while being stirred magnetically at 140 to 145°C for 2 hours under vacuum (5 mm). A well dispersed mixture of dry stigmasterol (268g, 649 mmol) and sodium methoxide (40 mg, 0.75 mmol) was added portionwise within 1 hour and the mixture was stirred at 170 to 175°C for an additional 21 hours. The reaction mixture was liberated from unreacted material either by column chromatography (2% diethylether in hexane on silicagel) or by a sequential extraction using two immissible solvents. The unreacted stigmasterol was precipitated upon addition of hexane and the solution was then filtered. The filtrate was extracted with methanol to remove unreacted starting oil material. TLC (hexane/diethylether/acetic acid (90:10: 1) gave an R_f equal to 0.71. The yield was 434 g (70 %). The ester product was a viscous oil.

When the experiment was repeated using freshly made sodium ethoxide, almost the same level of conversion was

obtained as with sodium methoxide. However, this was not seen with commercially available sodium ethoxide, which performed more poorly than sodium methoxide.

The procedure works also from a concentrated mixture of EPA and DHA omega-3 fatty acids in triglyceride form (EPAX™ 5500 TG, BioNova) with a similar yield of final product.

Example 2

The effect of a phytosterol-fish oil ester-containing diet on plasma lipid levels in guinea pigs.

Guinea pigs were chosen for this project, as their blood lipid profiles and responses to dietary manipulation more closely resemble those of humans than do more commonly used laboratory rodents. Two groups of eight guinea pigs each were fed a standard, non-purified guinea pig chow (Prolab guinea pig 5P18, PMI Nutrition International, Inc., Brentwood, MO). Baseline values for blood lipids were determined and then the animals were placed on a control diet (Group 1) or a phytosterol-fish oil ester-containing diet (Group 2).

Phytosterol-fish oil esters were prepared as described in Example 1 and mixed 5:1 with corn oil. This was incorporated into crushed chow to give a concentration of phytosterol-fish oil esters of 2.5% (w/w). Control diet was prepared using an equivalent amount of corn oil. Both control and test diets were supplemented with 0.08% cholesterol. The chow was re-pelleted using a Hobart extruder. Food was stored in sealed plastic bags with nitrogen purging at -20°C in the dark. Fresh food was prepared each week.

Blood samples were collected from each animal after 2 and 4 weeks for determination of plasma lipids (total cholesterol, HDL-cholesterol, non-HDL-cholesterol, and triacylglycerols).

Guinea pigs fed phytosterol-fish oil esters (2.5% g/100 gram diet) had significantly lower levels of plasma total cholesterol and triacylglycerol compared to control fed animals after 4 weeks of feeding (Table 1). At this time, plasma cholesterol and triacylglycerols were 36% and 29% lower in the treatment group. A statistically significant effect of phytosterol-fish oil esters on cholesterol was also evident after 2 weeks where the reduction was 30% compared to the control value. The changes in cholesterol level could be completely explained by changes in the amount of non-high density lipoprotein (HDL)-cholesterol (Table 2). Non-HDL cholesterol was 30% and 38% lower in the phytosterol-fish oil ester-fed group at 2 and 4 weeks, respectively, whereas there were no differences in HDL-cholesterol.

These results illustrate the ability of dietary phytosterol-fish oil esters to reduce the levels of plasma cholesterol and triacylglycerol. It is also shown that phytosterol-fish oil esters lower non-HDL cholesterol (*"bad cholesterol"*) but do not affect the level of HDL (*"good cholesterol"*).

Table 1.

The effect of a phytosterol/fish oil esters containing diet on plasma total cholesterol and triacylglycerol levels in guinea pigs

		Total Cholesterol	Triacylglycerol
Group 1	Week 2	1.72 ± 0.38	0.92 ± 0.26
	Week 4	2.05 ± 0.20	0.87 ± 0.16
Group 2	Week 2	1.22 ± 0.10 *	0.77 ± 0.22
	Week 4	1.32 ± 0.20 *	0.62 ± 0.13 *

Results are mean ± S.D. of 8 guinea pigs per group. The baseline values for plasma total cholesterol and triacylglycerol were 1.28 ± 0.12 (mM) and 0.65 ± 0.11 (mM) respectively.

*Significantly lower than the corresponding value for Group 1 ($p < 0.05$; Bonferroni's Multiple Comparison Test).

Table 2.

The effect of a phytosterol/fish oil esters containing diet on lipoprotein metabolism in guinea pigs

		HDL Cholesterol	non-HDL Cholesterol
Group 1	Week 2	0.14 ± 0.03	1.58 ± 0.4
	Week 4	0.16 ± 0.06	1.90 ± 0.2
Group 2	Week 2	0.11 ± 0.04	1.11 ± 0.14 *
	Week 4	0.16 ± 0.03	1.17 ± 0.23 *

Results are mean ± S.D. of 8 guinea pigs per group. The baseline values for HDL cholesterol and non-HDL cholesterol were 0.16 ± 0.07 (mM) and 1.14 ± 0.16 (mM) respectively.

*Significantly lower than the corresponding value for Group 1 ($p < 0.05$; Bonferroni's Multiple Comparison Test).

Example 3.

The effect of a phytosterol-fish oil ester-containing diet on plasma lipid levels in an obese rat model

The efficacy of a phytosterol-fish oil ester-containing diet to lower plasma triacylglycerol and cholesterol was studied in the JCR:La-cp (corpulent) rat, a genetic model of obesity (O'Brien and Russell, 1997). Animals of this strain, if homozygous for the autosomal recessive cp gene (cp/cp), are obese, insulin resistant, hyperinsulinemic, and highly hypertriglyceridemic. In addition the obese animals exhibit poor vascular responsiveness and develop ischemic lesions of the myocardium with age. Rats that are homozygous normal or heterozygous (+/?), are lean and metabolically normal. The effect of phytosterol-fish oil ester feeding was determined using obese (cp/cp) rats at 8 weeks of age, when the rats are clearly obese and fully insulin resistant. Lean litermates (+/?) of the obese animals were included in the study as benchmark for comparison. Obese animals were fed one of four diets: a control diet containing no added oil (Group 1); a control diet containing 2.6 g/kg canola (Group 2); or diets containing 0.5 or 2.6 g/kg phytosterol-fish oil ester (Group 3 and Group 4, respectively). The lean animals (Group 5) received the control without canola. The various test diets were fed for four weeks.

Preparation of the diets using standard rat chow (Rodent Diet 5001, PMI Nutrition International, St Louis, Mo) was essentially the same as described in Example 2. Phytosterol-fish oil ester was mixed with canola oil (5:1) and the oil mixture was added to the powered diet at a concentration of 0.5 g/kg or 2.6 g phytosterol ester/kg diet,

which was then pelleted. Control diets contained no added oil or 2.6 g/kg canola oil. Food was stored in sealed plastic bags with nitrogen purging and maintained at 4°C. Fresh food was prepared each week.

Blood samples were collected from each animal at the start and after 4 weeks for determination of plasma lipids (total cholesterol, cholesterol esters, phospholipids, and triacylglycerols).

Obese JCR-La rats exhibit marked hypertriglyceridemia and elevated plasma cholesterol levels compared to their lean littermates (Group 1 or 2 versus Group 5; Table 3). There was a concentration-dependent effect of dietary phytosterol-fish oil esters on plasma lipid concentrations. The lower dose of 0.5 g phytosterol-fish oil ester/kg food had no impact on lipid parameters in animals fed for 4 weeks (Group 3 versus Group 2 at 12 weeks; Table 3). However 2.6 g phytosterol-fish oil ester /kg food reduced triacylglyerol level from control levels by 51% (1.26 mM versus 2.59 mM in the control). Although this is a marked reduction, the animals are still strongly hypertriglyceridemic (Group 4 versus Group 5). There was also a modest reduction of cholesterol levels in animals fed the high dose of phytosterol-fish oil ester (13% reduction in total cholesterol; 17% reduction in cholesterol esters). There was a tendency for phospholipid values to be reduced in phytosterol-fish oil ester-fed animals but this did not reach statistical significance.

The results show that phytosterol-fish oil esters decrease plasma triacylglyerol and cholesterol in obese JCR-La rats and that this occurs in a dose-dependent manner. The reduction in triacylglycerol and cholesterol esters is

consistent with a substantial reduction in very low density lipoprotein (VLDL) particles through a decreased rate of VLDL production by the liver. These improvements in lipid profile might also be expected to have a beneficial effect on the insulin-resistant state of these animals.

Table 1. Whole serum lipid concentrations in high dose ON-1-treated male JCR-LA-cp rate

	Free Cholesterol	Cholesteryl esters	Total cholesterol	Phospholipids	Triacylglycerols
Initial values at 8 weeks of age:					
Group 1 (no oil control)	0.73 ± 0.11	1.19 ± 0.39	2.63 ± 0.49	2.19 ± 0.36	2.06 ± 1.19
Group 2 (oil control)	0.68 ± 0.10	1.89 ± 0.31	2.58 ± 0.40	2.01 ± 0.20	1.37 ± 0.63
Group 3 (0.5 mg/kg dose)	0.75 ± 0.12	2.01 ± 0.19	2.76 ± 0.30	2.35 ± 0.33	2.17 ± 1.11
Group 4 (2.6 mg/kg dose)	0.74 ± 0.09	1.94 ± 0.24	2.67 ± 0.33	2.28 ± 0.27	2.64 ± 0.84
Group 5 (lean control)	0.40 ± 0.06	1.31 ± 0.09	1.79 ± 0.12	1.01 ± 0.13	0.25 ± 0.16
Final values at 12 weeks of age:					
Group 1 (no oil control)	0.67 ± 0.06	1.58 ± 0.24	2.25 ± 0.29	1.92 ± 0.27	2.58 ± 0.93
Group 2 (oil control)	0.60 ± 0.09	1.61 ± 0.16	2.21 ± 0.23	1.87 ± 0.22	2.59 ± 0.58
Group 3 (0.5 mg/kg dose)	0.62 ± 0.14	1.55 ± 0.26	2.17 ± 0.37	1.90 ± 0.26	2.51 ± 0.71
Group 4 (2.6 mg/kg dose)	0.50 ± 0.06	1.34 ± 0.11**	1.92 ± 0.15*	1.66 ± 0.19	1.26 ± 0.72**
Group 5 (lean control)	0.34 ± 0.03	0.90 ± 0.04	1.24 ± 0.06	0.71 ± 0.04	0.17 ± 0.04

Values are mmol/l; mean ± S.D., 8 rats in each group. ** Significantly lower compared to group 2(P<0.05).

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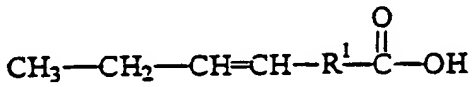
CLAIMS:

1. A nutritional supplement comprising an ester formed between a sterol and an omega-3 fatty acid for lowering cholesterol and triglyceride levels in the bloodstream of a subject.

2. The nutritional supplement according to claim 1, wherein the sterol is a phytosterol.

3. The nutritional supplement according to claim 1 or 2, wherein the omega-3 fatty acid has the formula:



wherein R^1 is a (C_3-C_{40})alkenylene group comprising at least one double bond.

4. The nutritional supplement according to claim 3, wherein R^1 has from 2 to 5 double bonds.

5. The nutritional supplement according to claim 4, wherein the omega-3 fatty acid is eicosapentaenoic acid 20:5ω3 (EPA).

6. The nutritional supplement according to claim 4, wherein the omega-3 fatty acid is docosahexaenoic acid 22:6ω3 (DHA).

7. The nutritional supplement according to any one of claims 1 to 6, wherein the sterol is a phytosterol.

8. The nutritional supplement according to any one of claims 1 to 7, wherein the sterol is stigmasterol.

9. The nutritional supplement according to any one of claims 1 to 7, wherein the sterol is sitosterol.

10. The nutritional supplement according to any one of claims 1 to 7, wherein the sterol is fucosterol.

11. The nutritional supplement according to any one of claims 1 to 7, wherein the sterol is fucostanol.

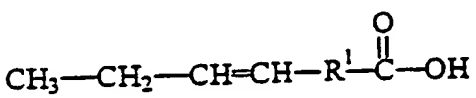
12. The nutritional supplement according to any one of claims 1 to 7, wherein the sterol is β-sitostanol.

13. The nutritional supplement according to any one of claims 1 to 12, further comprising an edible additive.

14. A method of lowering cholesterol and triglyceride levels in the bloodstream of a subject, the method including the step of administering to a subject an effective amount of a nutritional supplement comprising an ester formed between a sterol and an omega-3 fatty acid.

15. The method according to claim 14, wherein the omega-3 fatty acid is derived from fish oil.

16. The method according to claim 14 or 15, wherein the omega-3 fatty acid has the formula:



wherein R^1 is a (C_3-C_{40}) alkenylene group comprising at least one double bond.

17. The method according to claim 16, wherein R^1 has from 2 to 5 double bonds.

18. The method according to claim 17, wherein the omega-3 fatty acid is eicosapentaenoic acid 20:5ω3 (EPA).

19. The method according to claim 17, wherein the omega-3 fatty acid is docosahexaenoic acid 22:6ω3 (DHA).

20. The method according to any one of claims 14 to 19, wherein the sterol is a phytosterol.

21. The method according to any one of claims 14 to 20, wherein the sterol is stigmasterol.

22. The method according to any one of claims 14 to 20, wherein the sterol is sitosterol.

23. The method according to any one of claims 14 to 20, wherein the sterol is fucosterol.

24. The method according to any one of claims 14 to 20, wherein the sterol is fucostanol.

25. The method according to any one of claims 14 to 20, wherein the sterol is β-sitostanol.

26. Use of a nutritional supplement comprising an ester formed between a sterol and an omega-3 fatty acid, as defined in any one of claims 1 to 13, for lowering cholesterol and triglyceride levels in the bloodstream of a subject.

27. A foodstuff having a nutritional value enhanced by incorporation of the nutritional supplement according to any one of claims 1 to 13.

28. Use of the nutritional supplement according to any one of claims 1 to 13 in the manufacture of a foodstuff.

29. A process for preparing the nutritional supplement as defined in any one of claims 1 to 13, which comprises the step of reacting a sterol with an omega-3 fatty acid, or an ester thereof, in the presence of a base.

30. A process according to claim 29 wherein the base is a metal (C_1-C_{10}) alkoxide.

31. A process according to claim 30, wherein the metal (C_1-C_{10}) is sodium methoxide.

32. A process according to claim 29, 30 or 31, which further comprises the step of precipitating unreacted sterol with a suitable non-polar solvent, and filtering off the precipitated unreacted sterol to leave a filtrate.

33. A process according to claim 32, wherein the non-polar solvent is hexane.

34. A process according to claim 32 or 33, which further comprises the step of extracting the filtrate with a suitable immiscible solvent to remove unreacted omega-3 fatty acid, or an ester thereof, from the filtrate.

35. A process according to claim 34, wherein the immiscible solvent is methanol.

36. A process according to any one of claims 29 to 35, wherein the ester of the omega-3 fatty acid is a triglyceride ester.

37. A process according to any one of claims 29 to 35, wherein the ester of the omega-3 fatty acid is an ethyl ester.

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EXAMINER	
PUTTLITZ, KARL J	
ART UNIT	**PAPER NUMBER**
1621	

DATE MAILED: 09/01/2004

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No.	Applicant(s)	
	10/070,181	WRIGHT ET AL.	
	Examiner	Art Unit	
	Karl J. Puttlitz	1621	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *08 July 2002*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-37* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☒ Claim(s) *1-37* is/are allowed.

6)☐ Claim(s) _____ is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other: _____.

DETAILED ACTION

Claim Objections

Claims 8-13, 21-25, 32, 34, 36 and 37 are objected to under 37 CFR 1.75(c) as being in improper form because a multiple dependent claim cannot depend on another multiple dependant claim See MPEP § 608.01(n).

Claim Rejections - 35 USC § 112

The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 14, 15, 26, 27, 28, 29, 36, and 37 are rejected under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for the omega-3 fatty acids of claim 3 and particular phytosterols listed in the application does not reasonably provide enablement for all omega-3 fatty acids and sterols. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make or use the invention commensurate in scope with these claims.

The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to practice the invention commensurate in scope with these claims.

"The standard for determining whether the specification meets the enablement requirement [in accordance with the statute] was cast in the Supreme Court decision of *Mineral Separation v. Hyde,* 242 U.S. 261, 270 (1916) which postured the question: is the experimentation needed to practice the invention undue or unreasonable? That standard is still the one to be applied. *In re Wands*, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988). Accordingly, even though the statute does not use the term "undue experimentation," it has been interpreted to require that the claimed invention be enabled so that any person skilled in the art can make and use the invention without undue experimentation. *In re Wands*, 858 F.2d at 737, 8 USPQ2d at 1404 (Fed. Cir. 1988). See also *United States v. Telectronics, Inc.,* 857 F.2d 778, 785, 8 USPQ2d 1217, 1223 (Fed. Cir. 1988) ("The test of enablement is whether one reasonably skilled in the art could make or use the invention from the disclosures in the patent coupled with information known in the art without undue experimentation.").

In the instant case the rejected claims cover all omega-3 fatty acids and sterols. Based on the above standards, the disclosure must contained sufficient information to enable one skilled in the pertinent art to use this invention without undue experimentation. See M.P.E.P. 2164.01. Given the scope of the claims, it does not.

The specification and the examples do not provide sufficient disclosure that would provide one of ordinary skill guidance to practice the invention, given the infinite amount of all possible permutations of omega-3 fatty acids and

sterols. In this regard, the disclosure does teach those of ordinary skill how to select appropriate omega-3 fatty acids and sterols with the desired function of lowering cholesterol and triglyceride levels in the bloodstream., where the instant specification only describes broad geni of omega-3 fatty acids and sterols.

The examiner understands that there is no requirement that the specification disclose every possible embodiment if there is sufficient guidance given by knowledge in the art (See M.P.E.P. § 2164.05(a) "[t]he specification need not disclose what is well-known to those skilled in the art and preferably omits that which is well-known to those skilled and already available to the public. *In re Buchner*, 929 F.2d 660, 661, 18 USPQ2d 1331, 1332 (Fed. Cir. 1991); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986), *cert. denied*, 480 U.S. 947 (1987); and *Lindemann Maschinenfabrik GMBH v. American Hoist & Derrick Co.*, 730 F.2d 1452, 1463, 221 USPQ 481, 489 (Fed. Cir. 1984).").

However, the instant case goes beyond what is known in the art, because the specification does not offer any guidance on how one of ordinary skill would go about practicing the invention for all omega-3 fatty acids and sterols with the desired function of lowering cholesterol and triglyceride levels in the bloodstream, which would constitute undue experimentation.

Applicant is reminded of the heightened enablement for chemical inventions. Specifically, the amount of guidance or direction needed to enable the invention is inversely related to the amount of knowledge in the state of the art as well as the predictability in the art. *In re Fisher*, 427 F.2d 833, 839, 166

USPQ 18, 24 (CCPA 1970). The "amount of guidance or direction" refers to that information in the application, as originally filed, that teaches exactly how to make or use the invention. The more that is known in the prior art about the nature of the invention, how to make, and how to use the invention, and the more predictable the art is, the less information needs to be explicitly stated in the specification. In contrast, if little is known in the prior art about the nature of the invention and the art is unpredictable, the specification would need more detail as to how to make and use the invention in order to be enabling. [I]n the field of chemistry generally, there may be times when the well-known unpredictability of chemical reactions will alone be enough to create a reasonable doubt as to the accuracy of a particular broad statement put forward as enabling support for a claim. This will especially be the case where the statement is, on its face, contrary to generally accepted scientific principles. Most often, additional factors, such as the teachings in pertinent references, will be available to substantiate any doubts that the asserted scope of objective enablement is in fact commensurate with the scope of protection sought and to support any demands based thereon for proof. [Footnote omitted.]

Here, the requirement for enablement is not met since the claims go far beyond the enabling disclosure. Accordingly, base on the forgoing, the rejected claims are *prima facie*, non-enabled for their full scope.

Claims 14, 15, 26, 27, 28, 29, 36, and 37 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

The claims cover all esters of all omega-3 fatty acids and sterols.

To satisfy the written-description requirement, the specification must describe every element of the claimed invention in sufficient detail so that one of ordinary skill in the art would recognize that the inventor possessed the claimed invention at the time of filing. *Vas-Cath*, 935 F.3d at 1563; *see also Lockwood v. American Airlines, Inc.*, 107 F.3d 1565, 1572 (Fed. Cir. 1997) (patent specification must describe an invention and do so in sufficient detail that one skilled in the art can clearly conclude that "the inventor invented the claimed invention"); *In re Gosteli*, 872 F.2d 1008, 1012 (Fed. Cir. 1989) ("the description must clearly allow persons of ordinary skill in the art to recognize that [the inventor] invented what is claimed").

For chemical and biotech inventions, "[a]n adequate written description of a DNA ... 'requires a precise definition, such as by structure, formula, chemical name, or physical properties,' not a mere wish or plan for obtaining the claimed chemical invention." *Regents of the University of California v. Eli Lilly & Co.*, 119 F.3d 1559, 1566 (Fed. Cir. 1997), *cert. denied*, 523 U.S. 1089 (1998). Courts have stated that "[i]n claims involving [non-genetic] chemical materials, generic

formulae *usually indicate with specificity what the genericclaims encompass*. One skilled in the art can distinguish such a formula from others and *can identify many of the species* that the claims encompass. Accordingly, such a formula is normally an adequate description of the claimed genus." *Eli Lilly & Co.*, 119 F.3d 1568, (emphasis added). There is no such specificity here, since the claims merely recite all omega-3 fatty acids and sterols.

The terms "omega-3 fatty acids" and "sterols" contain almost no information by which a person of ordinary skill in the art would understand that the inventors possessed the entire scope of the claimed invention. At best, it simply indicates that one should test an infinite number of compounds in order to see if they posses the desired function of lowering cholesterol and triglyceride levels in the bloodstream.

Here the claims recite a copolymer of 2,2-dinitropropyl acrylate and 2,2-dinitrobutyl acrylate. However, the specification fails to describe any parameters of the claimed polymer, such as molecular weights, or other monomers that may be used in order to achieve the desiredenergetic binder. While the need for some experimentation is by no means necessarily fatal, "reasonable detail must be provided in order to enable members of the public to understand and carry out the invention.". Id. This reasonable detail is lacking in the captioned application. Specifically, the specification provides little guidance in the way of selecting a particular copolymers, or even of narrowing the range of molecular weights, or

co-monomers in order to find a suitable copolymer with the desired characteristics as an energy binder.

Nowhere does the specification point to any particular language in the application setting forth how to select those copolymers that would be likely candidates of the claimed formula. While it is true that a patent need not disclose that which is already well known in the art in order to be enabling, *see, e.g., Hybritech Inc. v. Monoclonal A* (Fed. Cir. 1986), *cert. denied*, 480 U.S. 947 (1987), far more is needed to enable the claimed copolymers. The specification fails to identify any suitable copolymers, or explain how one can discover such a copolymer which has the desired attributes of a an energy binder.

The following is a quotation of the second paragraph of 35 U.S.C. 112:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claims 1-37 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claim 7 does not further limit claim 1.

Claim 15 states that the omega-3 fatty acid is derived from fish oil. It is unclear exactly what compounds Applicant intends by this language since the structure of compounds "derived from fish oil" is indefinite.

Claims 26 and 28 are use claims which do not recite specific steps in the lowerinf of cholesterol and triglyceride levels, or for the manufacture of a food stuff. See vM.P.E.P. § 2173.05(q) ("Attempts to claim a process without setting

forth any steps involved in the process generally raises an issue of indefiniteness under 35 USC 112, second paragraph. For example, a claim which read: "A process for using monoclonal antibodies of claim 4 to isolate and purify human fibroblast interferon." was held to be indefinite because it merely recites a use without any active, positive steps delimiting how this use is actually practiced. *Ex parte Erlich*, 3 USPQ2d 1011 (Bd. Pat. App. & Inter. 1986). . . . Although a claim should be interpreted in light of the specification disclosure, it is generally considered improper to read limitations contained in the specification into the claims. See *In re Prater*, 415 F.2d 1393, 162 USPQ 541 (CCPA 1969) and *In re Winkhaus*, 527 F.2d 637, 188 USPQ 129 (CCPA 1975), which discuss the premise that one cannot rely on the specification to impart limitations to the claim that are not recited in the claim. ").

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (a) the invention was known or used by others in this country, or patented or described in a printed publication in this or a foreign country, before the invention thereof by the applicant for a patent.
>
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

Claims 1-3, 7, 9, 14-16, 20, 22, and 26-28 are rejected under 35 U.S.C. 102(a) as being anticipated by EP 897970, as evidenced by

counterpart U.S. Patent No. 6,160,886 to van Amerongen et al. (van Amerongen).

This patent teaches a process for the preparation of stanol fatty acid esters mixtures by inesterification of stanol fatty acid esters starting material is new and comprises reacting (at least 50%) saturated fatty acid groups of starting material with a source of at least one fatty acid moiety containing at least 35% (especially 45%) polyunsaturated fatty acid (PUFA) groups.

The product is incorporated into foods, See column 1, lines 58-64.

The products have blood cholesterol lowering effects. See column 4, lines 6-14.

The foregoing anticipates the claims within the meaning of section 102.

Claims 1-3, 7, 9, 14-16, 20, 22, and 26-28 are rejected under 35 U.S.C. 102(a) as being anticipated by EP 1004595 (EP 594).

This reference teaches phytosterols that are esterified with polyunsaturated fatty acids. The products are useful in foods. See claim 7.

The products are useful in lowering blood cholesterol levels. See paragraph 0025.

The foregoing anticipates the claims within the meaning of section 102.

Claims 1-3, 9, 14, 15, 16, 22 and 26-28 are rejected under 35 U.S.C. 102(b) as being anticipated by U.S. Patent No. 5,502,045 to Miettinen et al. (Miettinen).

This patent teaches fatty acid esters of sitostanol. See column 3, lines line 7-16. The product is useful as food to lower cholesterol. See paragraph bridging columns 4 and 5.

The foregoing anticipates the rejected claims.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

Claims 4-6, 8, 10-13, 17-19, 21 and 23-25 are rejected under 35 U.S.C. 103(a) as being unpatentable over each of Amerongen or EP 594.

The claims cover specific omega-3 fatty acids or sterols not explicitly disclosed by Amerongen or EP 594 so as to amount to anticipation (See M.P.E.P. § 2131: "[t]he identical invention must be shown in as complete detail as is contained in the ... claim." *Richardson v. Suzuki Motor Co.*, 868 F.2d 1226,

1236, 9 USPQ2d 1913, 1920 (Fed. Cir. 1989). The elements must be arranged as required by the claim, but this is not an *ipsissimis verbis* test, i.e., identity of terminology is not required. *In re Bond*, 910 F.2d 831, 15 USPQ2d 1566 (Fed. Cir. 1990).). However, based on the above, the applied references teaches the elements of the claimed invention with sufficient guidance, particularity, and with a reasonable expectation of success, that the invention would be *prima facie* obvious to one of ordinary skill (the prior art reference teaches or suggests all the claim limitations with a reasonable expectation of success. See M.P.E.P. § 2143).

Claims 29-37 are rejected under 35 U.S.C. 103(a) as being unpatentable over Miettinen in view of Lo et al., JAOCS, 60, 4, 1983 (Lo).

The claims are drawn to a process for preparing the nutritional supplement, which comprises reacting a sterol with an omega-3 fatty acid, or an ester thereof, in the presence of a base.

Miettinen teaches esterification of stanols and fatty acids. The difference between the process disclose by Miiettinen and the process covered by the rejected claims is that Miettinen fail to disclose the presence of a base. It is for this proposition that the examiner joins Lo. Specfically, Lo teaches the interesterification of fatty acids with sodium methoxide.

One of ordinary skill would have been motivated to modify Miettinen to include a base catalyst since Lo teaches that theses catalyst are useful for the

esterification of fatty acids. Therefore the rejected claims are prima facie obvious in view of the combination of Miettinen and Lo since these references suggest the elements of the claims with a reasonable expectation of success.

Conclusion

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Karl J. Puttlitz whose telephone number is (571) 272-0645. The examiner can normally be reached on Monday-Friday (alternate).

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Johann Richter can be reached on (571) 272-0646.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the receptionist whose telephone number is (703) 308-1235.

Karl J. Puttlitz
Assistant Examiner

S/ B.
ACTING FOR
Johann R. Richter, Ph.D., Esq.
Supervisory Patent Examiner
Biotechnology and Organic Chemistry
Art Unit 1621
(571) 272-0646

Notice of References Cited	Application/Control No. 10/070,181	Applicant(s)/Patent Under Reexamination WRIGHT ET AL.	
	Examiner Karl J. Puttlitz	Art Unit 1621	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-6,106,886	08-2000	van Amerongen et al.	426/611
	B	US-5,502,045	03-1996	Miettinen et al.	514/182
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N	1004594		EP		
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Lo et al., JAOCS, 60, 4, 1983
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

ATTORNEY DOCKET NO. 15113.0005U2
PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	
	)	
Wright *et al.*	)	Group Art Unit: 1621
	)	
Application Serial No.: 10/070,181	)	Examiner: Puttlitz, Karl J.
	)	
Filing Date: July 8, 2002	)	Confirmation No.: 3116
	)	
For: A NUTRITIONAL SUPPLEMENT FOR LOWERING SERUM TRIGLYCERIDE AND CHOLESTEROL LEVELS	)	

AMENDMENT AND RESPONSE TO OFFICE ACTION

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

NEEDLE & ROSENBERG, P.C.
Customer Number 23859

February 28, 2005

Sir:

Responsive to the Office Action mailed on September 1, 2004, please amend the application as set forth below and consider the remarks that follow. Also enclosed are a Supplemental Information Disclosure Statement and Form PTO-1449. Finally, enclosed is a Request for a Three-Month Extension of Time and Credit Card Form PTO-2038 in the amount of $1,200.00 ($1,020.00 to cover the extension of time fee and $180.00 to cover the Supplemental Information Disclosure Statement).

Amendments to the Specification begin on page 2 of this Amendment.

The **Listing of Claims** begins on page 3 of this Amendment.

Remarks begin on page 5 of this Amendment.

AMENDMENTS TO THE SPECIFICATION

On page 1 of the specification, after the title and before the first heading, please insert the following:

Cross Reference to Related Applications

The present application claims priority to U.S. Patent Application No. 09/385,834, filed August 30, 1999, which application is incorporated herein fully by this reference.

LISTING OF CLAIMS

This listing of claims will replace all prior versions, and listings, of claims in the application. A clean version of the pending claims is appended hereto for the Examiner's convenience.

Claims 1-37 (Cancelled)

Please add new claims 38-49.

38. (New) A process for preparing an ester comprising the step of reacting a sterol with an omega-3 fatty acid, wherein the omega-3 fatty acid comprises eicosapentaenoic acid 20:5ω3 (EPA), docosahexaenoic acid 22:6ω3 (DHA), an ester thereof, or a mixture thereof, and the sterol comprises stigmasterol, in the presence of a base.

39. (New) The process of claim 38, wherein the omega-3 fatty acid is eicosapentaenoic acid 20:5ω3 (EPA).

40. (New) The process of claim 38, wherein the omega-3 fatty acid is docosahexaenoic acid 22:6ω3 (DHA).

41. (New) The process of claim 38, wherein the omega-3 fatty acid comprises a mixture of eicosapentaenoic acid 20:5ω3 (EPA) and docosahexaenoic acid 22:6ω3 (DHA).

42. (New) The process of claim 38, wherein the ester of the omega-3 fatty acid is a triglyceride ester.

43. (New) The process of claim 38, wherein the ester of the omega-3 fatty acid is an ethyl ester.

44. (New) The process of claim 38, wherein the base is a metal (C_1-C_{10}) alkoxide.

45. (New) The process of claim 44, wherein the metal (C_1-C_{10}) is sodium methoxide.

46. (New) The process of claim 38, further comprising the step of precipitating unreacted sterol with a suitable non-polar solvent, and filtering off the precipitated unreacted sterol to leave a filtrate.

47. (New) The process of claim 46, wherein the non-polar solvent is hexane.

48. (New) The process of claim 46, further comprising the step of extracting the filtrate with a suitable immiscible solvent to remove unreacted omega-3 fatty acid, or an ester thereof, from the filtrate.

49. The process of claim 48, wherein the immiscible solvent is methanol.

REMARKS

Claims 1-37 have been cancelled. Claims 38-49 have been added. Claims 38-49 are pending.

Support for new claims 38-41 can be found in the specification at, *inter alia*, page 8, line 5, Example 1 at pages 15 and 16, and original claim 1. Support for new claims 42 and 43 can be found in the specification at, *inter alia*, page 8, lines 13-15 and original claims 36 and 37. Support for new claims 44 and 45 can be found in the specification at, *inter alia*, page 14, lines 10-12 and original claims 30 and 31. Support for new claims 46-49 can be found in the specification at, *inter alia*, page 14, line 27-page 15, line 13, and original claims 32-35.

No new matter has been added by the new claims; therefore, Applicants respectfully request that examination continue on the new claims.

A clean copy of all of the pending claims as they are believed to have been cancelled and added is attached to this Amendment as an appendix. The appended clean copy of all of the pending claims is provided only as a convenience to the Examiner and is not intended to be an amendment of the claims pursuant to 37 C.F.R. § 1.121.

I. Claim Objections

The Office Action has objected to claims 8-13, 21-25, 32, 34, 36, and 37 under 37 C.F.R. § 1.75(c) as being in improper form because a multiple dependent claim cannot depend on another multiple claim. Claims 1-37 have been cancelled, and new claims 38-49 have been added. New claims 38-49 are not multiple dependent claims; therefore, Applicant respectfully requests the objection be withdrawn.

II. Rejections under 35 U.S.C. § 112, First Paragraph

The Office Action has rejected claims 14, 15, 26, 27, 28, 29, 36, and 37 under 35 U.S.C. § 112, first paragraph, because "the specification, while being enabling for the omega-3 fatty acids of claim 3 and particular phytosterols listed in the application does not reasonably provide enablement for all omega-3 fatty acids and sterols." The Office Action further recites that "the

specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make or use the invention commensurate in scope with these claims."

Claims 1-37 have been cancelled; therefore, the rejection is moot. With respect to new claims 38-49, the application provides ample enabling disclosure for practicing invention. Example 1 of the application at pages 15 and 16 provides one aspect of the invention, which is a detailed synthetic procedure for producing stigmasterol esters of EPA and DHA. Based on the disclosure of the specification and, in particular, Example 1, one of ordinary skill in the art would not require undue experimentation to practice the invention as recited in new claim 39 and all claims dependent therefrom.

The Office Action has rejected claims 14, 15, 26, 27, 28, 29, 36, and 37 under 35 U.S.C. § 112, first paragraph, for failing to comply with the written description requirement. The Office Action asserts that "the claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one of ordinary skill in the art that the inventor(s), at the time the application was filed, had possession of the claimed invention."

Claims 1-37 have been cancelled; therefore, the rejection is moot. With respect to new claims 38-49, the written description requirement has been satisfied. Throughout the specification and, in particular, Example 1, it is clear to one of ordinary skill in the art that the inventors were indeed in possession of the invention at the time of filing as recited in new claims 38-49.

Therefore, Applicant asserts that the invention recited in new claims 38-49 satisfies all requirements of 35 U.S.C. § 112, first paragraph.

III. Rejections under 35 U.S.C. § 112, Second Paragraph

The Office Action has rejected claims 1-37 under 35 U.S.C. § 112, first paragraph. Claims 1-37 have been cancelled; therefore, the rejection is moot.

IV. Rejections under 35 U.S.C. § 102

The Office Action has made the following rejections under 35 U.S.C. § 102:

1. Claims 1-3, 7, 9, 14-16, 20, 22, and 26-28 under 35 U.S.C. § 102(a) in view of European Publication No. 897970;

2. Claims 1-3, 7, 9, 14-16, 20, 22, and 26-28 under 35 U.S.C. § 102(a) in view of European Publication No. 1004594; and

3. Claims 1-3, 9, 14-16, 20, 22, and 26-28 under 35 U.S.C. § 102(b) in view of U.S. Patent No. 5,502,045.

Claims 1-37 have been cancelled. Further, the rejections under 35 U.S.C. § 102 are not directed toward original claims 29-37, which recite a process for making sterol esters. Therefore, new claims 38-49, which are also method of making claims, are novel in view of the art.

V. Rejections under 35 U.S.C. § 103

The Office Action has rejected claims 4-6, 8, 10-13, 17-19, 21, and 23-25 under 35 U.S.C. § 103(a) in view of European Publication Nos. 897970 and 1004594. Claims 1-37 have been cancelled. Further, these rejections under 35 U.S.C. § 103 are not directed toward original claims 29-37, which recite a process for making sterol esters. Therefore, new claims 38-49, which are also method of making claims, would not have been obvious to one of ordinary skill in the art in view of European Publication Nos. 897970 and 1004594.

The Office Action also asserts that original claims 29-37 would have been obvious under 35 U.S.C. § 103 over U.S. Patent No. 5,502,045 to Miettinen in view of the journal article to Lo *et al.* In particular, the Office Action asserts that claims 27-39 are *prima facie* obvious in view of the combined teachings of Miettinen and Lo *et al.*

Claims 29-37 have been cancelled; however, for the sake of completeness, new claims 38-49 will be addressed in view of the combined teachings of Miettinen and Lo *et al.* It is well-established that, to establish *prima facie* obviousness of a claimed invention, all the claim limitations must be taught or suggested by the prior art. MPEP § 2143.03 (citing *In re Royka*, 490 F.2d 981, 180 USPQ 580 (CCPA 1974)). Said another way, claims for an invention are not

prima facie obvious if the cited reference does not suggest all elements of the claimed invention and the prior art does not suggest the modifications that would bring the primary references into conformity with the application claims. *In re Fritch*, 23 U.S.P.Q.2d, 1780 (Fed. Cir. 1992). *In re Laskowski*, 871 F.2d 115 (Fed. Cir. 1989). This is not possible when the claimed invention achieves more than what any or all of the prior art references allegedly suggest, expressly or by reasonable implication.

Here, the claimed invention as recited in new claims 38-49 includes features neither disclosed nor suggested by Miettinen or Lo *et al.* For example, new claim 38 recites, *inter alia*, the use of "an omega-3 fatty acid, wherein the omega-3 fatty acid comprises eicosapentaenoic acid 20:5ω3 (EPA), docosahexaenoic acid 22:6ω3 (DHA), an ester thereof, or a mixture thereof" to produce sterol esters.

Miettinen and Lo *et al.* do not disclose or suggest the use of eicosapentaenoic acid 20:5ω3 (EPA), docosahexaenoic acid 22:6ω3 (DHA), an ester thereof, or a mixture thereof. In the case of Miettinen, Miettinen discloses the esterification of rapeseed oil with β-sitstanol. There is no direction or motivation in Miettinen to use other fatty acids, particularly the omega-3 fatty acids EPA or DHA. Indeed, rapeseed oil does not even contain EPA and DHA. Similarly, Lo *et al.* only discloses the esterification of soybean oil with edible beef tallow. Similar to Miettinen, there is no suggestion or teaching in Lo *et al.* to use omega-3 fatty acids such as EPA or DHA, which are recited in new claims 38-49. Moreover, soybean oil also does not even contain DHA or EPA.

In the absence of any disclosure, teaching, or suggestion in Miettinen and Lo *et al.* to use the omega-3 fatty acids recited in new claims 38-49, the present invention would not have been obvious to one of ordinary skill in the art.

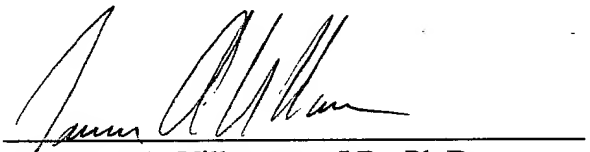
CONCLUSION

Pursuant to the above amendments and remarks, reconsideration and allowance of the pending application is believed to be warranted. The Examiner is invited and encouraged to directly contact the undersigned if such contact may enhance the efficient prosecution of this application to issue.

Enclosed is Credit Card Form PTO-2038 in the amount of $1,200.00 ($1,020.00 to cover the extension of time fee and $180.00 to cover the Supplemental Information Disclosure Statement). No further fee is believed to be due; however, the Commissioner is hereby authorized to charge any additional fees that may be required, or credit any overpayment to Deposit Account No. 14-0629.

Respectfully submitted,

NEEDLE & ROSENBERG, P.C.



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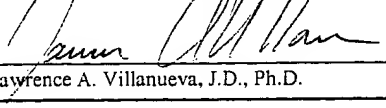

Lawrence A. Villanueva, J.D., Ph.D.

2/28/05
Date

APPENDIX

Clean Copy of All Pending Claims after Amendment (for the Examiner's convenience only)

What is claimed is:

38. A process for preparing an ester comprising the step of reacting a sterol with an omega-3 fatty acid, wherein the omega-3 fatty acid comprises eicosapentaenoic acid 20:5ω3 (EPA), docosahexaenoic acid 22:6ω3 (DHA), an ester thereof, or a mixture thereof, and the sterol comprises stigmasterol, in the presence of a base.

39. The process of claim 38, wherein the omega-3 fatty acid is eicosapentaenoic acid 20:5ω3 (EPA).

40. The process of claim 38, wherein the omega-3 fatty acid is docosahexaenoic acid 22:6ω3 (DHA).

41. The process of claim 38, wherein the omega-3 fatty acid comprises a mixture of eicosapentaenoic acid 20:5ω3 (EPA) and docosahexaenoic acid 22:6ω3 (DHA).

42. The process of claim 38, wherein the ester of the omega-3 fatty acid is a triglyceride ester.

43. The process of claim 38, wherein the ester of the omega-3 fatty acid is an ethyl ester.

44. The process of claim 38, wherein the base is a metal (C_1-C_{10}) alkoxide.

45. The process of claim 44, wherein the metal (C_1-C_{10}) is sodium methoxide.

46. The process of claim 38, further comprising the step of precipitating unreacted sterol with a suitable non-polar solvent, and filtering off the precipitated unreacted sterol to leave a filtrate.

47. The process of claim 46, wherein the non-polar solvent is hexane.

48. The process of claim 46, further comprising the step of extracting the filtrate with a suitable immiscible solvent to remove unreacted omega-3 fatty acid, or an ester thereof, from the filtrate.

49. The process of claim 48, wherein the immiscible solvent is methanol.

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